THE ROLE OF DENDRITIC CELLS IN THE PATHOGENESIS OF LEISHMANIASIS

by

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in this or any other university.

The data presented in this thesis is the work of the author, and to the best of my knowledge and belief, has not been previously written or published by any other person, except where acknowledged.

Pamela Konecny

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A second minor clinical study is included in this manuscript. I was fortunate to have had the opportunity to partake in a field study trialing a novel therapeutic agent in the treatment of cutaneous leishmaniasis in a hyperendemic area in Syria. The study was initiated by Dr Robert Davidson, in collaboration with Professor Hratch Balaban, Department of Dermatology, University of Aleppo, Aleppo, Syria. The work was supported by a Director's Initiative grant from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. Tragically, Professor Balaban died whilst the manuscript was in preparation. To work with Professor Balaban and his colleagues in the Department of Dermatology, University of Aleppo, was an enriching and enlightening experience. Colleagues, Professor Nigel Benjamin, Pharmacology Department, St Bartholomew's, London and Drs Simon Croft and Vanessa Yardley, London School of Hygiene and Tropical Medicine developed and tested the nitric oxide releasing creams in vitro and in vivo in mice. I was involved in the design, implementation and data analysis of the human study

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ABSTRACT

In humans, leishmaniasis comprises a spectrum of disease from cutaneous lesions to destructive mucocutaneous erosions or an insidiously progressive fatal visceralising disease. An estimated 1 to 1.5 million people are afflicted each year with cutaneous leishmaniasis and 500 000 victims succumb to visceral disease per annum world-wide. Leishmaniasis is caused by protozoan parasites, *Leishmania,* which are transmitted by the phlebotomine sand fly to the host where they become obligate intracellular parasites of mononuclear phagocytes. The protozoa are dimorphic. The flagellated promastigote (PM) form of the parasite from the sandfly inoculum enters the host cell where it transforms into a compact amastigote (AM) without flagellum in a parasitophorous vacuole (PV) within 24 hours and replicates by binary fission. Whether infection remains asymptomatic or develops into locally aggressive or disseminated disease is based in part on parasite type and virulence factors but predominantly on the host immune response.

Cell mediated immunity is required for control of infection following transmission of the parasite. This is dependent upon the type of interaction which occurs between the antigen presenting cells (APC), the first host cells to encounter the parasite and T helper (Th) cells.

Parallels with human leishmaniasis can be drawn from the murine model of *Leishmania major* infection. In genetically inbred mice there is distinct Th cell differentiation, characterised by their cytokine profile, which correlate with susceptibility or resistance to disease. The events which direct Th differentiation in

susceptible and resistant mouse strains remain controversial but are believed to operate in the first hours or days of infection. There is a body of evidence to suggest that dendritic cells (DC), the most potent APC, play a role in leishmaniasis. However their interaction had only been investigated with AM, not the initial infectious PM stage. Whether DC interact with PM and if so, whether this interaction plays a significant role in the primary immune response to *Leishmania*, particularly in Th differentiation, is a tantalising but as yet unanswered question.

Two studies are described in this thesis. The major study involved original laboratory research investigating the role of dendritic cells in the pathogenesis of leishmaniasis. The supplementary study was a clinical field study undertaken in an hyperendemic area for cutaneous leishmaniasis (CL) in Syria to trial a novel topical therapy for treatment of CL.

The major study was based on the development of a murine in-vitro model to investigate the hypothesis that DC internalise *L.major* PM and play a pivotal role in the early events that shape the development of a cellular immune response to infection. Conditions were defined for optimal DC internalisation of *L.major* PM and their stimulation of naive T cell responses to leishmanial antigens. Their potential to influence Th development, by cytokine production or modulation of co-stimulator expression, was then investigated.

The original findings from this research suggest that DC play an important role in establishing an early primary T cell response to *L.major* PM. DC internalise *L.major* PM with maximal expression of co-stimulatory and MHC Class II molecules and in

response produce IL-12. Combined with results of the primary stimulation assays these findings suggest that DC from both susceptible and resistant mouse strains are able to stimulate autologous naive T cells when pulsed with PM antigens and provide a cytokine environment predicted to favour the development of a Th1 response. The importance of DC in the overall immune response to *Leishmania* is further underlined by observations that macrophages appear to be temporarily paralysed during infection with *L.major* PM, particularly in their production of IL-12, due to a number of evasion strategies which the parasite has evolved. This work also identifies a potential use of DC in the search of non-peptide antigens shed by *L.major* which are presented via non-classical MHC pathways, as potential vaccine candidates.

The supplementary study involved the development and clinical study of a nitric oxide (NO) generating cream in the topical treatment of CL. CL often has a protracted period of healing, for months and occasionally years, with potential for secondary bacterial infection and significant disfigurement. Current first line treatment entails repeated intralesional injections which are painful, time consuming, expensive and now associated with increasing parasite resistance. Improvement in treatment is greatly needed.

Nitric oxide synthesised by macrophages is lethal to *Leishmania*. Since NO diffuses into tissues, we reasoned that NO-generating creams applied topically to lesions might be an effective and inexpensive treatment for CL. The theory that NO can be generated non-enzymatically by the acidification of nitrite (KNO_2) by a weak acid such as ascorbic acid (ASC), potassium chloride (KCL) or salicylic acid (SAL).

Initial laboratory experiments performed by colleagues demonstrated efficacy of this topical combination therapy in vitro and in mice. We designed the clinical study to trial the nitrite cream in combination with one of the three weak acids for four weeks in 40 patients with CL in Aleppo, Syria, and assess clinical responses. Only 11(28%) of 40 patients showed improvement and only 5 (12%) of 40 were cured at 2 months.

The investigation was small but demonstrated reasonably good patient tolerability and ease of application. Given the results of this study, and the urgent need for new, more easily applicable therapy, further development of NO-generating creams is warranted with assessment in placebo-controlled randomised trials.

Both studies have been published in good peer-reviewed journals advancing scientific knowledge in both pathogenesis and treatment of leishmaniasis. Both areas are inevitably linked and advances made in the former will hasten the development and refinement of much needed treatments and ultimately, prevention of disease.

PUBLICATIONS

Konecny P, Stagg AJ, Jebbari H, English N, Davidson RN and Knight SC. Murine dendritic cells internalise *Leishmania major* promastigotes, produce IL-12p40 and stimulate primary T cell proliferation in vitro. Eur J Immunol 1999;29:1803-1811

Davidson RN, Yardley V, Croft SL, Konecny P and Benjamin N. A topical nitric oxide-generating therapy for cutaneous leishmaniasis. Trans Roy soc Trop Med Hyg 2000;94:319-322

Davidson RN and Konecny P. Immunology and treatment of the leishmaniases. Curr Op Infect Dis. 1995; 8:336-341

PRESENTATIONS

Konecny P, Stagg AJ, Knight SC, Davidson RN. Murine dendritic cells internalise *Leishmania major*, produce IL-12 and stimulate primary T cell proliferation. 38th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC). Poster presentation. September 1998;Abstract No. G-25

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ABBREVIATIONS

ALM	autoclave-killed *L.major*
AM	amastigote
BSA	bovine serum albumin
CD80	cell differentiation molecule 80
CL	cutaneous leishmaniasis
CM	compete medium
Con A	concanavalin A
DTH	delayed type hypersensitivity
DC	dendritic cell
DCL	disseminated cutaneous leishmaniasis
DMSO	dimethylsulphoxide
EM	electron microscopy
FACS	fluorescent activated cell scan
FCS	fetal calf serum
GM-CSF	granulocyte macrophage colony stimulating factor
iNOS	inducible nitric oxide synthase
IFN-γ	interferon-gamma
IL-2	interleukin-2
LACK	Leishmania homologue of receptor for activated C kinase antigen
LDC	low density cell
LC	Langerhans cell
LM	light microscopy
LNC	lymph node cell

LPG	lipophosphoglycan
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
NK	natural killer cell
NLDC-145	nonlymphoid dendritic cell antibody-145
NLDC-b	biotinylated NLDC-145
NO	nitric oxide
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PKDL	post kala-azar dermal leishmaniasis
PM	promastigote
PV	parasitophorous vacuole
rIL-12	recombinant IL-12
SLA	soluble leishmanial antigen
TGF-β	transforming growth factor-beta
TCR	T cell receptor
TNF-α	tumour necrosis factor-alpha
VL	visceral leishmaniasis

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CHAPTER 1

BACKGROUND AND AIMS

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1.1 Overview

*Leishmania*sis is predominantly a zoonosis occurring in tropical and sub-tropical regions, encompassing a range of clinical syndromes. The diseases caused by the protozoan parasite, *Leishmania* were described in antiquity (Peters and Killick-Kendrick 1987). The "Balkh sore" was the name given to cutaneous *Leishmania* lesions in Central Asia as early as the first century AD. Medieval Moslem scholars referred to it as the "oriental sore" in 15th century records. The parasite was first isolated by Leishman (a Scotsman) in 1903 post-mortem from the spleens of British soldiers in India who succumbed to an idiopathic febrile illness. He recognised their morphological similarity to trypanosomes. In the same year Donovan described the characteristic intracellular parasitic forms, later named Leishman-Donovan bodies, from the splenic aspirate of a patient. The phlebotomine sandfly was subsequently identified as the insect vector in 1942 (reviewed by Peters and Killick-Kendrick 1987).

The past three decades has witnessed an escalating interest in *Leishmania*sis. World Health Organisation (WHO) included it as one of the six items in the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases in 1976. An estimated 1 to 1.5 million people are afflicted each year with cutaneous *Leishmania*sis and 500 000 victims succumb to visceral disease per annum world-wide (Desjeux 1996). There is an increasing incidence of leishmaniasis with major epidemics occurring in India (Addy and Nandy 1992), Bangladesh (Elias, et al 1989),

Sudan (Seaman, et al 1996) and Brazil (Jeronimo, et al 1994). Visceral leishmaniasis is emerging as an opportunistic pathogen in conditions where cell mediated immunity is compromised such as HIV infection (Alvar, et al 1997) and organ transplantation (Berenguer, et al 1998). Leishmaniasis is an increasing threat to non-immune travellers and military personnel in regions of endemicity (Magill, et al 1993).

The treatment of visceral leishmaniasis is less than ideal. The limited drugs available have significant toxicity and high failure relates due to parasite resistance and persistence (reviewed by Davidson and Konecny 1995, Berman JD 1997). There is an urgent need for better understanding of the parasite and its interaction with the host's defence system for improved control and management of diseases caused by *Leishmania*.

In humans, *leishmaniasis* comprises a spectrum of disease from cutaneous lesions to destructive mucocutaneous erosions or an insidiously progressive and often fatal visceralising disease (reviewed by Sacks, et al 1993). The phlebotomine sandfly transmits the parasite between mammalian hosts. *Leishmania* are dimorphic protozoa. The flagellated promastigote (PM) form of the parasite from the sandfly inoculum enters the host cell where it becomes an obligate intracellular parasite of mononuclear phagocytes. It transforms into a compact amastigote (AM) without flagellum in a parasitophorous vacuole in the host cell within 24 hours and replicates by binary fission. Whether infection remains asymptomatic or develops into locally aggressive or disseminated disease is based in part on parasite virulence factors but mainly on the host immune response (Reiner and Locksley 1995).

Cell mediated immunity is required for control of infection following transmission of the parasite. This is dependent upon the type of interaction which occurs between the antigen presenting cells (APC), the first host cells to encounter the parasite and T helper (Th) cells. Parallels with human leishmaniasis can be drawn from the murine model of *Leishmania major* infection (Reiner and Locksley 1994). Indeed, major advances in immunological research into host resistance and susceptibility to disease have occurred in the past decade using this model (reviewed by Locksley, et al 1999). In genetically inbred mice there is distinct differentiation of specific Th cells, characterised by their cytokine profile, which correlate with susceptibility or resistance to *Leishmania* infection. The events which direct Th differentiation in susceptible and resistant mouse strains remain controversial but are considered to operate in the first hours to days following infection (Launois, et al 1997).

Until recently there was a dearth of knowledge of the type of host cell which initially encounters the leishmanial parasite and provides the bridge between the innate and acquired immune response. Dendritic cells (DC) are highly potent APC which are essential for initiating a cellular immune response. A growing body of evidence suggests DC have a major role in the host response to intracellular pathogens (reviewed by Reis e Sousa, et al 1999). During natural infection, one of the earliest interactions with the host immune system will be between sandfly-transmitted PM and cells resident in skin. Initial work found dermal macrophages remained relatively inert upon infection with PM (Locksley, et al 1988). Macrophages have little respiratory oxidative burst and the usual production of cytokines required for intracellular killing and activation of the cellular immune response, particularly interleukin-12 (IL-12), is inhibited due to a number of evasion strategies which the

parasite has evolved (Reiner, et al 1994; Carrera, et al 1996). Cells of the DC lineage including Langerhans' cells (LC) in the epidermis and dermal DC were alternative contenders for *Leishmania* parasitism. To date LC have been shown to take up *L.major* AM and effectively stimulate *Leishmania*-specific T cell responses (Moll, et al 1995). However, minimal uptake of PM by LC was observed in their study. Whether dermal DC interact with *Leishmania* PM and influence naïve Th differentiation at the initial stage of infection, was considered a crucial question requiring clarification.

A murine in vitro model was therefore developed to investigate the hypothesis that DC internalise *L.major* PM and play a pivotal role in the early events that shape the development of a cellular immune response to *Leishmania* infection. The development of this in vitro model was undertaken by the author as a pilot project under the supervision of Professor Stella Knight and Dr Andrew Stagg in the Antigen Presentation Research Group and Dr Robert Davidson, Department of Infection and Tropical Medicine, Northwick Park Hospital, Harrow. This work was successful in attracting funding from the Wellcome Trust to continue with the research for a two year period, the results of which form the basis of this thesis.

Conditions were defined for optimal DC internalisation of *L.major* PM. The potential for *Leishmania* exposed DC to influence Th development was then investigated with primary stimulation assays and measurement of cytokine production and costimulator expression. The potential role of a recently characterised DC receptor, DEC-205 (Jiang, et al 1995), homologous to the mannose receptor macrophages use to endocytose *Leishmania* PM, was also studied in the DC uptake of *L.major* PM.

Soluble *L.major* antigen was further purified from culture supernatants to determine whether non-peptide antigens could be presented by DC via recently described non-classical pathways, employing CD1 presentation and stimulation of naïve T cell responses (Porcelli, et al 1992).

The findings from this research suggest that DC play an important role in establishing an early primary T cell response to *L.major* PM. DC internalising *L.major* maximally express costimulatory and MHC Class II molecules and produce IL-12. Combined with results of the primary stimulation assays, these findings suggest that DC from both susceptible and resistant mouse strains are able to stimulate autologous naive T cells when pulsed with *L.major* PM and *L.major* antigens from PM cultures and provide a cytokine environment predicted to favour the development of a Th1 response. Further, semi-purified, non-peptide *L.major* PM antigen was capable of stimulating naïve T cell proliferation when presented by DC. This work also identifies a potential role for DC in the search for non-peptide antigens shed by *L.major* which are presented via non-classical MHC pathways. These may provide potential antigens for vaccine development.

1.2 *Leishmania* – the parasite

1.2.1 Life cycle

Leishmania are dimorphic protozoa, existing as extracellular, motile, 10-15μm flagellated PM at 25°C in the gut of the female phlebotomine sandfly and as 2-3μm intracellular AM without exteriorised flagellum in cells of reticuloendothelial origin

in humans and other susceptible mammals. The PM has a major surface glycoconjugate, lipophosphoglycan (LPG), which constitutes a dense glycocalyx that covers the entire surface of the parasite including the flagellum (Handman and Goding 1985). As the PM matures in the insect midgut, the structure of the LPG undergoes modification enabling the mature forms, termed metacyclics, to migrate to the sandfly proboscis for transmission (Fig 1.1). In addition to the role LPG play in macrophage attachment discussed below, it is immunogenic for host protective responses (Moll and Rollinghoff 1991) and has been identified as a virulence factor using LPG1 knock-out mice (Späth, et al 2000).

The sandfly feeds on pooled blood arising from repeated skin probing and highly infectious PM which occlude the proboscis of the feeding sandfly are left in the dermis. PM in the tissue spaces activate complement via the alternate pathway by binding C3b, forming C3a, C5a and other chemotaxins for monocytes in the inflammatory exudate (Brittingham and Mosser 1996). [1] The elongated, altered LPG on metacyclic PM confers resistance to complement-mediated lysis by virtue of the dense glycocalyx forcing C5b-9 membrane attack complex formation to occur too far from the surface to allow insertion into the parasite membrane (Peuntes, et al 1990). Another parasite surface glycoprotein, gp63, a membrane protease also protects the PM by cleaving C3b to a form that cannot fix the membrane attack complex (Brittingham and Mosser, 1996). Phagocytosis of PM by macrophages involves recognition of specific parasite ligands by receptors on the macrophage surface. LPG fixes predominantly C3b, facilitating phagocytosis of PM by macrophages via the complement receptor CR1 (Da Silva, et al 1989). By internalising via CR1 and CR3

[1] In humans, the major mechanism for PM invasion appears to be through the engagement of CR1 on erythrocytes and the classical complement pathway (Dominguez and Torana 1999).

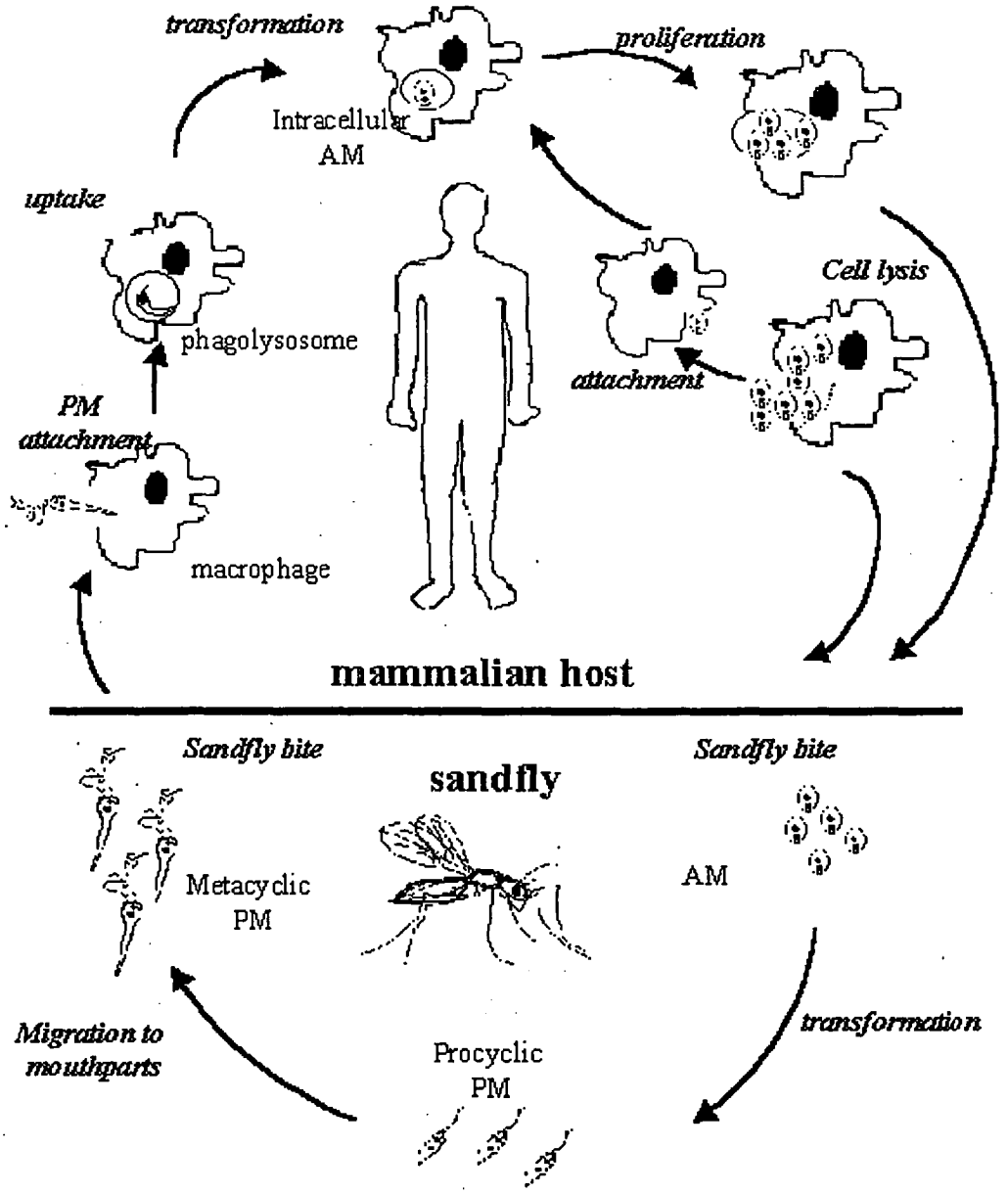


Fig1.1 Developmental cycle of *Leishmania*. In mammalian hosts, metacyclic PM transmitted by sandfly bite are internalised by macrophages. Intracellular development from PM to AM occurs within the phagolysosome. AM replicate within the macrophage until cell rupture occurs and released AM attach to recruited macrophages to persist within the host. AM within cells or from ruptured cells are internalised during sandfly feeding on the host. In the sandfly vector procyclic forms develop in the midgut and transform into metacyclic forms as they migrate into the proboscis.

macrophage receptors, which do not trigger the respiratory burst, parasite survival is enhanced. LPG also protects the PM by scavenging hydroxyl radicals and superoxide ions normally released upon activation of NADPH oxidase by phagoctyosis (Bogdan and Rollinghoff 1999).

Other parasite surface glycoproteins, such as gp63 (Russell and Wilhelm 1986) and additional macrophage receptors such as CR3, the mannose-fucose receptor (Wilson and Pearson 1988), the Fc receptor, the fibronectin receptor (Wyler, et al 1995) and the receptor for advanced glycosylation end products (Mosser, et al 1987) may also play a role in parasite internalisation. Phagocytosis then occurs by one of the following mechanisms. Classical 'zipper-like' phagocytosis occurs when a pseudopod extends from the surface membrane and engulfs the particle by advancing along the edge like a zipper. A more recently described process of complex pseudopodia coiling and stacking, termed 'coiling phagocytosis' directs the organism to cytoplasmic compartments (Bogdan and Rollinghoff 1999).

Components of sandfly saliva may enhance infectivity, predominantly by inhibiting macrophage function, but are not necessary to establish infection in the host (reviewed by Solbech and Laskay 2000).

Following attachment to the macrophage, the PM are rapidly phagocytosed and reside in parasitophorous vacuoles which then fuse with secondary lysosomes to form a phagolysosome (Sacks, et al 1993). The PM shed their LPG coat, lose their flagellum and roundup into 2-3μm AM forms.

The AM able to survive lysosomal enzyme attack and low pH replicate in the phagolysosome by binary fission until the burgeoning macrophage ruptures allowing the spread of the AM to adjacent macrophages which have been recruited to the site by the inflammatory response. Uptake of AM by macrophages is more efficient than PM internalisation and appears to occur primarily through macrophage FcR and CR3 receptors (Guy and Belosevic 1993) and heparan sulfate proteoglycans (Love, et al 1993). By entering either macrophages of the reticuloendothelial organs or of the skin where they continue to divide, AM are thus responsible for maintaining and spreading infection within the host. Ingestion of infected macrophages from the circulation or skin by the feeding phlebotomine sandfly completes the life cycle (Fig 1.1).

Sandflies of the genus *Lutzomyia* transmit leishmaniasis in the Americas and *Phlebotomus* transmit leishmaniasis elsewhere (Peters and Killick-Kendrick 1987). Mammalian reservoirs, depending on *Leishmania* species and geographical location are predominantly primate, canine and rodent species. Man is an incidental host, but may also serve as a reservoir as occurs in India where there is extensive human cutaneous disease. In the Old World, sandfly breeding requires an arid warm environment where larvae grow in the cool humid microclimate of cracks and crevices in the walls of dwellings or rodent burrows. Cases typically occur in dry, rocky hill country where the particular mammalian reservoirs, such as the gerbil and domestic dog live in close proximity to man. In the New World, sandfly larvae prefer the leaf mould of the forest floor and forest rodents and incidental forest visitors are the mammalian host (Peters and Killick-Kendrick 1987).

1.2.2 Classification

The parasite species is difficult to determine microscopically. There is no defined sexual stage so traditional criteria for species identification cannot be applied. Speciation was originally based on the pattern of human disease caused by the parasite, its geographic distribution, specific animal reservoirs and species of sandfly vector. Assays using isoenzyme electrophoresis, species-specific monoclonal antibodies, restriction endonuclease digestion of kinetoplast DNA (kDNA) and species-specific hybridisation with kDNA probes (reviewed by Pearson, et al 2000) have enabled more accurate speciation creating a taxonomy currently in a state of flux.

For practical purposes, the major syndromes of visceral, cutaneous, and mucocutaneous *Leishmania*sis are divided into Old World and New World and attributed to the corresponding species (Table 1.1). In brief, visceral leishmaniasis (VL) is caused by *L.donovani* in the Old World, *L.infantum* in the Mediterranean littoral, and *L.chagasi* and *L.amazonensis* in the New World. *L. major* and *L.tropica* are the commonest cause of cutaneous leishmaniasis in the Old World and *L.mexicana* and *L.braziliensis* in the New World. Mucocutaneous disease occurs only in the New World and is caused by *L.braziliensis* (Lainson and Shaw 1987).

Table1.1 Leishmania taxonomic table (adapted from Lainson and Shaw, 1987)

Disease	Species	Geographical distribution
		Old World
Visceral leishmaniasis	*L.donovani*	Indian subcontinent, N&E.China, Pakistan, Nepal, E.Africa
	L.infantum	Middle East, Mediterranean littoral, Balkans, C&SW.Asia, N&W.China, N& sub-Saharan Africa
	L.chagasi	Latin America
	L.amazonensis	Brazil
	L.tropica (rare)	Middle East, Saudi Arabia, India, C&WAsia, N.Africa Pakistan, Mediterranean littoral
	L.spp	Kenya, Ethiopia, Sudan, Somalia
Post-kala-azar dermal leishmaniasis (PKDL)	*L.donovani*	Indian subcontinent
Cutaneous leishmaniasis	*L.major*	Middle East, India, Pakistan, N&W.China
	L.tropica	Mediterranean littoral, Middle East, N.Africa, India, Pakistan, C&W. Asia
	L.aethiopica	Ethiopian highlands, Kenya, Yemen
	L.infantum	Middle East, Mediterranean, C.Asia, N&W.China, N.&sub-saharan Africa
	L.donovani	E.Africa
Diffuse cutaneous leishmaniasis (DCL)	*L.aethiopica*	Ethiopian highlands, Kenya, Yemen
		New World
Cutaneous leishmaniasis	*L.mexicana*	C&S.America, Texas
	L.amazonensis	Amazon basin, Brazil
	L.pifanoi	Venezuela
	L.garnhami	Venezuela
	L.venezuelensis	Venezuela
	L. braziliensis	C&S.America
	L.guyanensis	Guyana, Surinam, N.Amazon basin
	L.peruviana	Peru
	L.panamensis	Panama, Costa Rica, Colombia
	L.colombiensis	Colombia, Panama
	L.chagasi	C&S.America
Diffuse cutaneous leishmaniasis	*L.amazonensis*	Amazon basin, Brazil
	L.pifanoi	Venezuela
	L.mexicana	C&S.America, Texas
	L.spp	Dominican Republic
Mucosal leishmaniasis	*L.braziliensis*	C&S.America

1.3 Leishmaniasis – human disease

1.3.1 Clinical

The clinical manifestations of leishmaniasis depend on the species and virulence factors of *Leishmania* and host immune responses, which are partly genetically determined.

Visceral leishmaniasis is the clinical syndrome where the parasites phagocytosed by macrophages in the skin eventually disseminate to the mononuclear phagocytes throughout the reticuloendothelial system resulting in hepatosplenomegaly, bone marrow failure and often death (Fig 1.2). The term kala-azar or black sickness derives from the characteristic deepening skin pigmentation in those with dark skin. The original inoculation site usually goes unnoticed or bears a transient papule. The spleen becomes massively enlarged as splenic lymphoid follicles are replaced by parasitised mononuclear cells and Kupffer cells in the liver expand in size and frequency many of them containing AM. Diagnosis is usually confirmed with direct smear and Geimsa stain of splenic or bone marrow aspirate (Fig 1.3).

In cutaneous disease a papule forms, enlarges and eventually ulcerates at the inoculation site on exposed areas of the body. The incubation period may vary from weeks to months or even years. The Old World cutaneous ulcers are generally benign with morphology dependent on the parasite responsible. *L.tropica* is usually urban with smaller, single, drier ulcers while *L.major* occurs in more rural locales and the "wet" lesions are usually larger and more ulcerative (Fig 1.4).

Fig 1.2. An African patient infected with *L.donovani* with typical features of kala-azar, massive splenomegaly, wasting and hyperpigmentation. (Photograph courtesy of Dr R.N. Davidson)

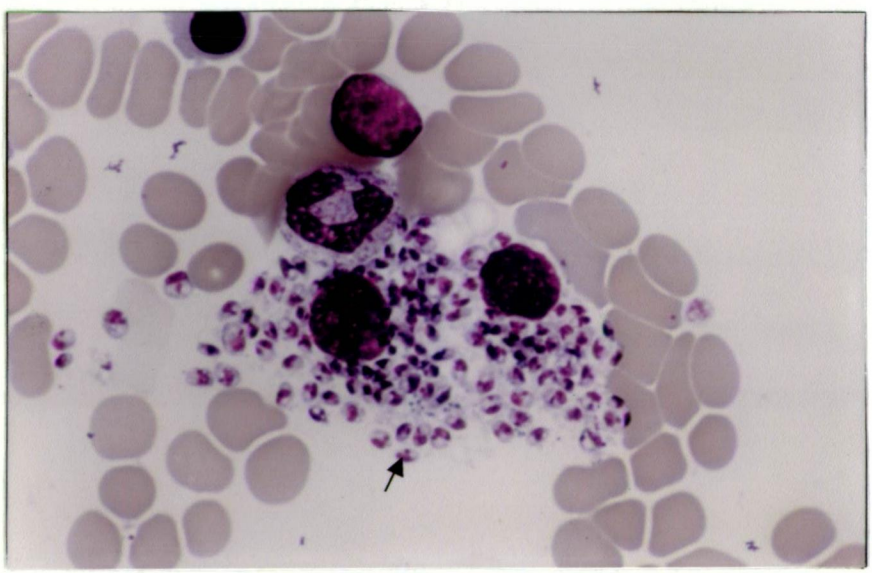


Fig 1.3. Geimsa stain of a bone marrow aspirate from a *L.donovani* infected patient with VL. Macrophages are packed with AM and rupture easily on slide preparation. The AM display typical "dot-dash" appearance (arrow) of the kinetoplast (dot) and nucleus (dash).

In New World CL, *L.braziliensis* and *L.mexicana* also cause large, moist ulcers usually in rural or forest workers. All ulcers are typically painless, full skin thickness with a rolled edge and bed of granulation tissue. They heal spontaneously, usually within a year, leaving a papery, depigmented, depressed, burn-like scar (Pearson, et al 2000).

In a subgroup of people, *L.braziliensis* leads to subsequent destructive facial mucocutaneous disease months to years after the cutaneous ulcer has healed. The soft and cartilaginous tissues of the nose, palate and upper pharynx are eroded as a result of metastatic spread of the parasite following a primary cutaneous ulcer at a distant site. This condition termed espundia is often erosive and disfiguring and may lead to death through secondary bacterial infection (Fig 1.5).

The species are not restricted to causing either cutaneous, mucocutaneous or visceral disease. Different strains of *L.infantum* called *L.infantum* variants or zymodemes may cause cutaneous or mucocutaneous lesions rather than kala-azar (Ben-Ismail, et al 1992). *L.tropica* caused viscerotropic disease in American military personnel during the Gulf War (Magill, et al 1993). *L.donovani* may cause extensive cutaneous disease referred to as post-kala-azar dermal leishmaniasis (PKDL) following treatment of VL. In this condition multiple nodules, some containing parasites, appear over the body surface within a few years of apparent cure of VL. A rarer condition called diffuse cutaneous leishmaniasis (DCL) may complicate cutaneous ulceration, with multiple skin nodules over face and extremities teeming with parasites (Fig 1.6.A). Leishmaniasis recidivans is another disfiguring form of CL with satellite non-

Fig 1.4 Typical large, moist ulcer of *L.major* occurring on an exposed area of a Syrian man living on the outskirts of Aleppo.

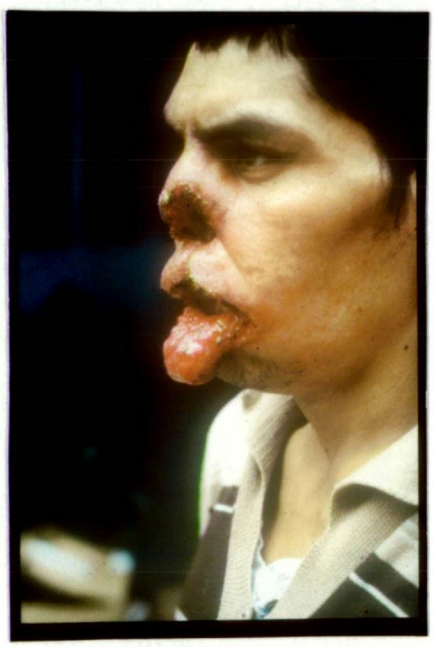


Fig 1.5 A Brazilian rubber plantation worker infected with *L.braziliensis* has the severely disfiguring mucocutaneous facial erosions referred to as espundia. (Photograph courtesy of Dr R N. Davidson.)

A B

Fig 1.6 A. An Ethiopian woman with diffuse cutaneous leishmaniasis (DCL) poorly responsive to therapy. (Photograph courtesy of Dr R.N.Davidson.) B. A young Syrian woman with leishmania recidivans affecting her arm and face who has undergone treatment with intralesional injections for 4 years. The lesion has a typical healing centre with satellite nodules.

ulcerative nodules around a healing centre which may take up to 20 years to resolve (Fig 1.6.B).

Interestingly, DCL resembles the lepromatous form of leprosy where the immune response is defective in containing parasites and massive numbers are found in lesions. Whereas PKDL and leishmaniasis recidivans reflect the tuberculoid end of the spectrum being a parasite-poor hyperergic variant of CL. The immunopathogenesis of these conditions are discussed in more detail below.

1.3.2 Host defence

Cell-mediated immunity plays a greater role than the humoral immune response in *Leishmania* infection. Cure of leishmaniasis correlates with the development of specific delayed type hypersensitivity (DTH), a proliferative lymphocyte response to *Leishmania* antigen and generally a life-long immunity to rechallenge with the same parasite (reviewed by Sacks, et al 1993). At the non-self curing end of the spectrum, VL is characterised by widespread dissemination of the parasite in the host's reticuloendothelial system with absent or reduced generalised DTH and T cell proliferative response. DCL which manifest as discrete nodules disseminated over the face and extremities is characterised by a loss of specific DTH and lymphocyte responsiveness to *Leishmania* antigen. In this condition the parasites proliferate and disseminate unchecked by the host's immune system. Cure of VL and DCL correlates with the restoration of DTH and lymphocyte responsiveness in-vitro. T cell function plays a central role in determining the host immune response to infection with *Leishmania* as outlined further below.

VL and DCL are characterised by absence of T cell interferon-γ (IFN–γ) and interleukin–2 (IL-2) production in response to *Leishmania* antigen in-vitro (Carvalho, et al 1985). Macrophages infected with *Leishmania* have decreased capacity to express major histocompatibility complex class II (MHC) (Reiner, et al 1988) and to produce IL-1 (Ho, et al 1992). Natural killer (NK) cell activity is also deficient in VL patients (Harms, et al 1991). An excess of IL-4 in sera and IL-10 from bone marrow cells (Karp, et al 1993) has also been detected in patients with VL. Furthermore neutralising antibodies to IL-10 and to IL-4 restores T cell proliferation and IFN–γ production in-vitro (Carvalho, et al 1994). Similarly, IL-12 can restore T cell responses to *Leishmania* in vitro from peripheral blood mononuclear cells (PBMC) of VL patients (Bacellar, et al 1996). These cytokines and their interactions are discussed in more detail below, but suffice to say that clear differences in T cell function occur in the various forms of human leishmaniasis.

L.major antigen has been detected in APC in the skin lesions and draining lymph nodes of patients suffering from CL (Elhassan, et al 1994). The involvement of APC and specific T cell responses, characterised by their cytokine profiles, are closely reflected in the experimental murine model of leishmaniasis which provides the opportunity for more detailed analysis of host response to infection with *Leishmania*.

1.4 Leishmaniasis - Murine Model

The murine model of leishmaniasis correlates well with human disease and has enabled major advances in the study of the immune response to this infection in the past two decades (Reiner and Locksley 1994). Although certain factors may influence the outcome of infection, such as the parasite strain, dose and site of inoculation, host sex, pregnancy and acquired immunosuppression, the most important factor is that of genetically determined host predisposition (reviewed by Blackwell 1996). A genetic locus for susceptibility to *L.major* (Scl-1) has been mapped to a region of mouse chromosome 11, which contains several candidate genes known to affect leishmanial infections (such as those encoding for iNOS, IL-4, interferon-regulatory-factor-1, small cytokine family and putative IL-12 response gene). Other potential loci for susceptibility have also been identified on chromosome 10 and 17 and are being characterised (Lipoldova M et al 2000). Using the mouse *L.major* model, it is appropriate to first outline what is understood of the innate immune response prior to exploring the acquired immune response.

1.4.1 Macrophages

Macrophages are the host cell in which *Leishmania* readily replicate. PM evading the parasiticidal activity of macrophages transform into AM. The AM stage has evolved various mechanisms to enhance survival. AM selectively and actively sequestrate and degrade MHC class II antigens (Antoine, et al 1998). AM metabolism is adapted to the acidic environment of the parasitophorous vacuole (Zilberstien and Shapira 1994). Structural surface proteins and glycolipids appear to

render AM less susceptible to proteolysis and hydrolysis (reviewed by Handman 1999). Another mechanism for parasite persistence involves inhibition of apoptosis through induction of 'pro-survival' cytokines such as GM-CSF, TNF-α and IL-6, thus prolonging the life of the host cell (Moore and Matlashewski 1994).

1.4.2 IFN-γ

Macrophage activation by T cell derived cytokines is required to contain infection. Both *scid* and *nude* mice succumb to infection unless T cell reconstituted (Mitchell 1983; Holaday, et al 1991). IFN-γ is one of the cytokines of critical importance. Resistant strains of mice have been rendered susceptible to fatal disease progression by targeted disruption of either the IFN–γ or the IFN–γ receptor gene. Recombinant IFN-γ has enabled clearance of *L.major* from infected macrophages from susceptible mice in-vitro (reviewed by Reiner and Locksley, 1995).

The parasiticidal effect of IFN–γ is primarily mediated through its positive influence on macrophage production of nitric oxide (NO) as demonstrated in Fig. 1.7. The final step in elimination of the parasite is mediated by NO. Macrophages treated with NO inhibitors are unable to restrict *L.major* replication in vitro and resistant mice are unable to control infection in vivo when given NO inhibitors (Liew, et al 1990). The macrophages of IFN–γ and IFN–γ receptor gene knock out mice are unable to produce NO. IFN-γ activates macrophage inducible nitric oxide synthase production (iNOS) to catalyse the oxidation of L–αrginine to NO (Fig 1.7) and it is the only cytokine independently capable of doing this (Stenger, et al 1994).

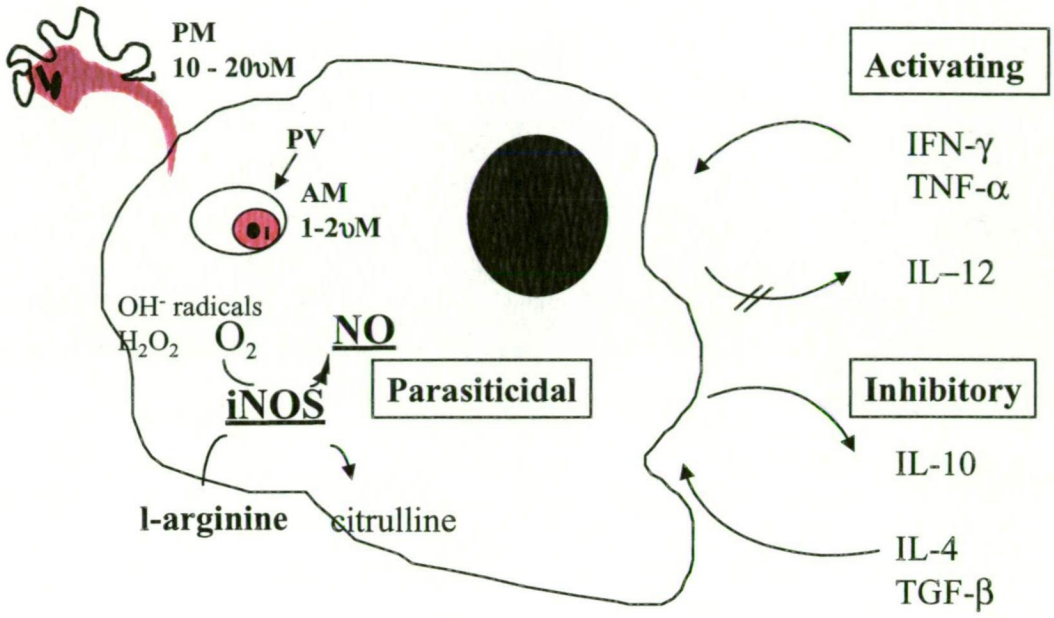


Fig 1.7 Schematic representation of macrophage processing of *Leishmania.* The PM is taken up by macrophages at initial host infection and transforms into an AM stage within a parasitophorous vacuole (PV). The final pathway for parasite killing is mediated by NO produced from iNOS oxidation of L-arginine. The predominant macrophage activating cytokines are IFN-γ and TNF-α produced by T cells and NK cells. IL-4 and TGF-β are important T cell derived inhibitory cytokines. The infected macrophages produce IL-10 which enhance T cell production of the inhibitory cytokines in the murine model. PM infection of the macrophage suppresses IL-12 secretion.

Other cytokines synergise with IFN–γ in NO production. TNF–α, in particular, can ameliorate disease in susceptible mice when administered in recombinant form (Titus, et al 1989). Neutralising anti-TNF antibody transiently exacerbated disease in resistant mice strains while minimally affecting the course of infection in BALB/c mice. Disruption of the p55 TNF receptor, which transduces the TNF–α response, in a genetically resistant background developed a persistent low-level infection with *L.major* that was, however, different to that which occurred in either the BALB/c mice or IFN–γ deficient mice. Other cytokines which also synergise with IFN–γ to activate macrophages, probably by sharing the IL-2 receptor γ chain, include IL-2, IL-4 and IL-7 (reviewed by Reiner and Locksley, 1995).

Transforming growth factor beta (TGF-β) on the other hand is a potent inhibitor of iNOS. TGF–β is prominent in non-healing lesions in susceptible mice and conversely is lacking in lesions and draining lymph nodes in resistant mice where it was inversely associated with iNOS production (Stenger, et al 1994). Another family of cytokines, IL-3, IL-5 and GM-CSF, which share a common signal-transducing molecule, also exacerbate *L.major* infection in vitro and in vivo (reviewed by Reiner and Locksley, 1995).

Once the parasite is degraded in lysosomes, antigen reappears at the cell surface as processed peptide associated with MHC class II molecules which can then be recognised by the T cell receptor (TCR) on primed CD4+ T cells. The intracellular events which take place after endocytosis are gradually being characterised. Within minutes of phagoctyosis, *Leishmania* are found in phagosomal compartments which mature and fuse with endocytic organelles to form a parasitophorous vacuole (PV)

containing lysosomal hydrolases, cathepsins and β-glucuronidase (Lang, et al 1994). A series of events occur within the PV such that it matures with time into a late endosomal compartment bearing lysosomal-associated membrane proteins LAMP-1 and LAMP-2 in addition to other markers. MHC class II molecules are acquired by the PV within 5-24 hours (Lang, et al 1994). In fact, these LAMP-positive compartments rich in MHC class II molecules constitute major class II peptide-loading compartments in APC. Co-localisation of *Leishmania* with MHC class II molecules within these compartments enables access to the host class II pathway. It is this cellular compartmentalisation which explains the association of *Leishmania* with host class II recognition in contrast to the class I recognition of other intracellular pathogens such as viruses.

Until recently there was a long held assumption that the macrophage was the only cell which internalised *Leishmania* and was thus responsible for initiating and maintaining the host's cellular immune response. However, macrophages are inefficient at priming naïve T cells. Resting macrophages do not express MHC class II molecules on their cell surface unless activated by IFN–γ and other cytokines (reviewed by Overath and Aebischer 1999). Furthermore, escape mechanisms have been recently identified during the initial PM stage of the parasite which appear to subdue the macrophages' initial role in immune defence. Usually, following phagocytosis of pathogenic organisms, macrophages secrete a range of activating cytokines such as IL-1, IL-12, TNF–α, acute phase stimulants such as IL-6, modulatory factors such as IL-10, haemopoetic stimulating factors such as M-CSF, GM-CSF and chemokines. Interestingly, following experimental infection of bone marrow derived macrophages from susceptible and resistant mice with *L.major*

metacyclic PM in-vitro, there was minimal transcription of these factors, in particular, IL-12, MIP-1α, TNF–α, IL-10 and iNOS (Reiner, et al 1994). The lack of early IL-12 induction following *L.major* infection of mice was also observed in vivo. This contrasts with the readily inducible IL-12 and TNF–α observed in macrophages infected with other pathogenic organisms, such as *Toxoplasma gondii*, *Listeria monocytogenes* and mycobacteria (reviewed by Reiner, et al 1995). *L.major* AM on the other hand stimulate immediate macrophage transcription of IL-12 and TNF–α (Green, et al 1990). Not only are PM relatively sheltered in their initial compartmentalisation within the macrophage but the host cell, unable to prime naïve T cells, also cannot send the usual signals to activate the cellular arm of the immune response. It is due to these important observations that other cell types, such as DC with their unique property of priming naïve T cells, warrant further investigation.

1.4.3 T helper cell differentiation into Th1 and Th2 subsets

Central to the cell mediated immune response required for intracellular parasite eradication are the lymphocyte subset of CD4+ T helper (Th) cells. Initial studies with murine CD4+ T cell clones demonstrated the existence of two sub-populations of Th cells, termed type 1 (Th1) and type 2 (Th2) based on mutually exclusive patterns of cytokine secretion in response to stimulation with concanavalin A (conA) or with antigen. Th1 subsets produce the cytokines IFN–γ, IL-2, and TNF–β which activate macrophages to kill intracellular parasites and effect DTH (Mosmann, et al 1986). Th2 cells produce IL-4, IL-5, IL-6 and IL10 and provide B lymphocyte help, particularly in the generation of IgG1 and IgE (Snapper, et al 1988).

Shortly after the recognition of two distinct subsets of T helper cells, studies using genetically inbred mice infected with *L.major* demonstrated the existence of these subsets in vivo (Locksley, et al 1987; Scott 1991; Boom, et al 1990). BALB/c mice susceptible to progressive disease with *L.major* infection secrete IL-4 in draining lymph node cells whereas resistant mice, including C57/BL6, CH3 and CBA strains release IFN–γ and lymphotoxin in regional lymph nodes. Thus the non-healing phenotype correlates with a Th2 response while Th1 differentiation occurs in those mice able to contain infection and self-cure. Subsequently it was shown that the capacity of *Leishmania*-specific CD4+ T cells to passively transfer disease resistance or susceptibility to immune depleted naïve mice correlated with the host production of Th1 or Th2 cytokine patterns (reviewed by Reiner and Locksley 1995).

Extensive research has focussed on the mechanism that mediates differentiation of these disparate Th subsets in mice of different genetic backgrounds. Initially, parasite antigens, particularly peptide epitopes from gp63, a conserved membrane protease immunogenic in most strains of mice, were investigated with the hope of finding a Th1-specific epitope that may serve as potential vaccine candidates (Jardim, et al 1990). Another potentially protective antigen, the LACK antigen (*Leishmania* homologue of receptor for activated C kinase) was identified and used in recombinant form in susceptibility studies as discussed below. Rather than show antigen driven T cell differentiation, these experiments in fact demonstrated that both Th1 or Th2 responses could arise from the same antigen depending on the conditions during priming. Using limiting dilution techniques and transgenic mice, it has been established that naïve T cells (Th0) have the capacity to differentiate into either Th1 or Th2 types depending on the cytokine milieu at the time of priming (Fig 1.8). IL-4

plays an important role in mediating Th2 development and IL-12 in driving Th1 development from naïve precursors. It appears that the Th differentiation pathway is decided within the first few days (Leiby, et al 1993), if not hours (Launois, et al 1995) of antigen/APC exposure.

Subsequent work has highlighted the importance of the innate immune system in determining the role of T cell responses. Physical parasite containment correlated with T cell-independent production of IFN-γ within the first 24 hours post infection in resistant mice (Laskay, et al 1995). NK cells were shown to be an important source of early IFN-γ. Furthermore, IL-12 strongly enhanced NK cell activity.

Important research has derived from the observation that the mouse phenotype could be altered by a range of immunological interventions. The susceptible BALB/c strain could be made resistant to infection with *L.major* and conversely resistant strains rendered susceptible by the addition of immunomodulating agents such as exogenous cytokines or cytokine inhibitors. Importantly all interventions were only effective if given very early in the course of infection. These studies, outlined in more detail below, have further delineated the specific contribution of individual cytokines.

1.4.4 IL-4

In the mouse phenotype susceptible to *Leishmania*, IL-4 excess appears to be more important in driving Th2 development than does the lack of IFN–γ production.

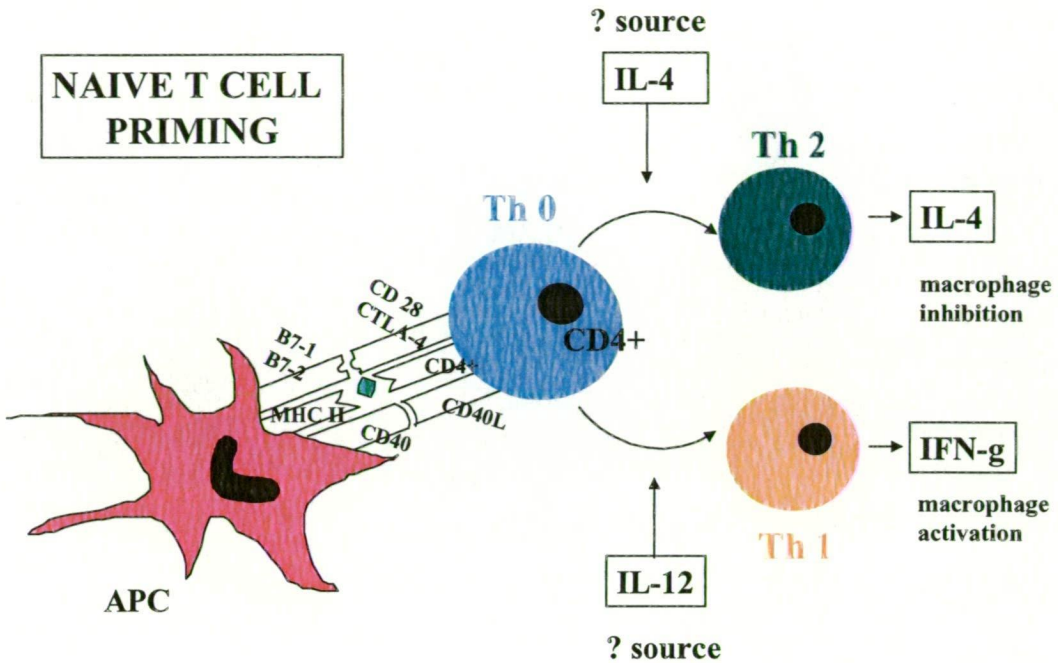


Fig 1.8. Schematic representation of the Th1/Th2 paradigm. The APC presents an antigenic peptide (shaded diamond) to the naïve T cell (Th0) in the context of MHC class II peptide. Costimulatory molecules on the surface of the APC, B7-1 or B7-2 and CD40, are required to activate the T cell. The balance of these costimulatory molecules and the predominant cytokine milieu, IL-12 or IL-4, directs the T cell differentiation pathway towards Th1 or Th2 development respectively.

The severity of disease correlates better with the presence of IL-4 than the lack of IFN–γ production (Morris, et al 1993). IFN–γ added to parasite inoculum significantly reduces the level of IL-4 production, but IFN–γ treatment at inoculation and for up to six weeks could not reverse the progression of disease (Scott 1991). On the other hand anti-IL-4 antibodies administered to susceptible mice cured *Leishmania* infection (Sadick, et al 1990). Additionally Il-4 has a predominantly inhibitory effect on the parasiticidal function of macrophages (reviewed by Bogdan and Rollinghoff, 1996).

So from where does early IL-4 arise? This question has provoked much controversy. In various experimental models several sources such as γδ T cells, eosinophils and cells of mast cell/basophil lineage have been proposed (reviewed by Launois, et al 1997). Data from several sources point towards CD4+ T cells alone being sufficient for the production of IL-4 necessary for Th2 differentiation (reviewed by Reiner and Locksley, 1995). Of the potential CD4 + candidates, naïve CD4+ cells were found to be capable of driving a Th2 response although the levels of IL-4 were very low and alone were not sufficient to account for this (Hsieh, et al 1993). Resting CD4+ memory cells produced significant IL-4 in response to challenge with antigen in vivo but minimally in vitro (Bradley, et al 1993). Furthermore this source would require the existence of a common environmental or cross-reactive self antigen. A subset of CD4+ T cells which express the NK1.1 antigen gained significant attention and was initially thought to provide the answer (Arase, et al 1992). These NK1.1+CD4 T cells are recent thymic emigrants which are MHC class I restricted and use a highly restricted repertoire of TCR. In vivo experiments with anti-CD3 rapidly invoked significant IL-4 responses in spleen although they comprised only 5% of the spleen

CD4+ population. As there is generally a lack of spleen cell stimulation in vitro it was suggested that these cells may be recruited to the spleen or lymph nodes following activation. However neither of these observations or speculation could be substantiated. The TCR on these NK1.1+ CD4+ cells have a Vα14 chain paired with Vβ8, Vβ7 or Vβ2 and accumulation of early IL-4 transcripts could not be demonstrated in the subset of CD4+T cells with these particular Vβ chains. Furthermore, NK1.1+ deficient mice still develop disease progression with a Th2 response and an absolute requirement for MHC class II has already been shown (reviewed by Reiner and Locksley, 1995).

Another CD4+ subset has been identified as the most likely source of early IL-4. This subset of CD4+ T cells bears the heterodimer Vβ4Vα8 receptor (Launois, et al 1997). Expansion of CD4 cells bearing these receptors following infection revealed recognition by these cells of specific parasite antigen. This antigen was cloned and designated LACK (*Leishmania* homologue of receptor for activated C kinase). Subsequently, recombinant LACK antigen has been shown to induce IL-4 mRNA after 16 hours in the same restricted population of Vβ4Vα8 CD4+ T cells in BALB/c but not C57BL/6 mice. The specific epitope recognised by I–a^d molecules was mapped to an 18-amino acid determinant of the LACK protein. Vβ4Vα8 deficient BALB/c mice injected with the LACK antigen did not produce IL-4 and were able to control infection. Similarly, transgenic BALB/c mice rendered LACK tolerant by MHC Class II associated LACK expression in the thymus developed a Th1 response and were resistant to infection with *L.major* (Julia, et al 1996). Thus it appears that the IL-4 required for Th2 development and susceptibility to *L.major* is produced by a restricted population of Vβ4Vα8 CD4+ T cells after cognate interaction with a single

antigen from the parasite. Whether the LACK antigen from *Leishmania* is solely responsible for the early IL-4 response or whether other antigens are recognised is unclear. The LACK antigen is unlikely to constitute a "Th2 epitope" as a small but detectable amount of IFN–γ mRNA is expressed when resistant mice are stimulated with this antigen and immunisation with LACK and IL-12 can prevent the Th2 response and protect BALB/c from subsequent infection.

Just as the individual effects of certain cytokines were becoming established and researchers began using "knockout" mice with selective inactivation of immunomodulatory genes to further delineate their function, conflicting data were reported by two groups on the role of IL-4. Using IL-4 knockout mice (IL-$4^{-/-}$) of BALB-c background, the susceptibility to *Leishmania* infection was reversed in one study (Kopf, et al 1996) but surprisingly unaffected in another very similar study (Noben-Trauth, et al 1996). These differences were puzzling but attributed in part to differences in the parasite strain used in these experiments. Subsequent work using IL-4Rα-deficient mice established that there probably are alternate IL-4 independent mechanisms for mediating a Th2 response and disease exacerbation. Another cytokine, IL-13 with similar properties to IL-4 which binds to the IL-4-Rα in addition to IL-13 receptors, was one such proposed mechanism (Noben-Trauth, et al 1999). Interestingly, in these and other studies IFN-γ production was unaltered in the IL-4 knockout mice and the level of IFN-γ did not correlate with disease progression, indicating an absence of cross talk between Th1 and Th2 cells in the *L.major* mouse model. Another hypothesis suggests that cell-intrinsic differences, specifically the ability of T cells to respond to the cytokine milieu, particularly IL-12, may be significant (Guler, et al 1996). This is discussed further below.

1.4.5 IL-12

Interleukin-12 (IL-12) is produced largely by phagocytic cells but also by B lymphocytes and dendritic cells in response to bacterial or parasitic infection (reviewed by Trinchieri 1995). It is a 70 kDa (p70) heterodimeric molecule comprising two covalently linked chains of 40 (p40) and 35 (p35) kDa. The p35 gene is constitutively expressed, usually at low levels, in most cell types whereas the p40 gene expression is restricted to cells producing IL-12p70. From the cells that produce the biologically active IL-12p70, free IL-12p40 is produced in excess of the IL-12p70 form in a ratio between 10:1 to 50:1. When human PBMC are stimulated with bacteria or bacterial products, IL-12 is produced up to levels of 10ng/ml of p40 and 1ng/ml of p70. IL-12 is similarly produced in mice, both in vivo and in vitro following stimulation of PMBC by bacteria, LPS and intracellular parasites (Gazzinelli, et al 1993). However recombinant IL-12p40 homodimers have been shown to inhibit the action of IL-12p70 (Mattner, et al 1993). It is important to consider these observations when reviewing IL-12 studies, where the original assays measured IL-12p40 rather than the more difficult to measure IL-12 p70.

Other cell types producing IL-12 in the murine model have been identified. Keratinocytes are minor producers (Goodman, et al 1994) and mast cells, depending on their tissue location and exposure to different cytokines, can be induced to produce IL-12 (Smith, et al 1994). Dendritic cells were then shown to produce IL-12 (Macatonia, et al 1995) as discussed in detail below.

IL-12 appears to be an important bridging cytokine between innate and acquired immunity. In brief, IL-12 early in infection stimulates NK cells and T cells to produce IFN–γ, which is important for activation and migration of phagocytic cells (Fig 1.8). IL-12 induces the production of other cytokines from these two cell types, TNF–α, GM-CSF, M-CSF, IL-3, IL-8 and IL-2. IFN–γ induction, however, is probably its most physiologically relevant function. IL-12 is a growth factor for preactivated T and NK cells and enhances cytotoxicity of $CD8^+$ T cells and NK cells. The production of IL-12 is regulated by other cytokines. It is enhanced by IFN–γ and GM-CSF and down-regulated by IL-4, IL-10 and TGF–β. In addition, TNF–α and IL-1 synergise with IL-12 to induce IFN–γ production (reviewed by Trinchieri 1995).

Perhaps most important is the role of IL-12 in driving differentiation of CD4+ cells along the Th1 pathway in response to antigen. *Listeria monocytogenes*-stimulated macrophages, a well studied model of bacterial infection, produce IL-12 and induce a Th1 response which is completely blocked with neutralising anti-IL-12 antibodies (Hsieh, et al 1993).

IL-12 plays a critical role in leishmaniasis. In the murine model of cutaneous leishmaniasis, IL-12 stimulates Th1 cell differentiation and enhances NK cell cytotoxicity (Scharton-Kersten, et al 1995). Susceptibility to infection with *Leishmania* in BALB/c mice can be reversed if IL-12 is administered in vivo within the first week of infection. Conversely, anti-IL-12 treatment of resistant mice confers a susceptible phenotype. This induced susceptibility can be restored by simultaneous treatment with anti-IL-4 (Heinzel, et al 1995) demonstrating important

feedback mechanisms for cytokine control. In addition IL-12 can inhibit IL-4 mRNA production even in the absence of IFN–γ (Wang, et al 1994).

Both resistant and susceptible strains of mice are able to mount an IL-12 response to infection with *L.major* PM in vivo. Similar levels of IL-12p40 could be detected by ELISA in the supernatants of the draining lymph nodes of both susceptible (BALB/c) and resistant (C3H) strains of mice as early as 24 hours following footpad inoculation with *L.major* PM (Sypek, et al 1993).

There is a suggestion that it is not an IL-4 effect but a lack of T cell responsiveness to IL-12 that confers susceptibility in BALB/c mice (Guler, et al 1996). In their in vitro system BALB/c T cells became unresponsive to IL-12 within about a week as a result of some intrinsic property rather than the effect of any cytokine produced within the system. In particular, one mouse strain, B10, appears be more sensitive to IFN–γ up-regulation of the IL-12 receptor.

The source of initial IL-12 production in *Leishmania* infection is unclear. As described above, macrophages only produce IL-12 when infected with the later AM stage of the parasite and not following PM infection. Recently, production of bioactive IL-12 by DC has been demonstrated and the role of IL-12 in mediating Th1 development and IFN–γ production has been recognised (Macatonia, et al 1995).

Table 1.2 Source and action of relevant cytokines (adapted from Locksley and Wilson 2000)

Cytokine	Principle cell source	Major biological effect
IL-2	T lymphocytes	T-cell proliferation, co-factor for B cell proliferation & Ig production
IL-4	T cells, mast cells, basophils	Proliferation B & T cells; facilitates IgG1,IgE synthesis; inhibits macrophage activation; antagonises Th1
IL-5	T cells, mast cells	Promotes antibody production, especially IgM, IgA, IgE, eosinophil expansion and activation; expansion T cell independent B cells
IL-6	Many types; mononuclear phagocytes, T cells, endothelial cells	Acute phase reactant; B cell proliferation & Ig production; T cell proliferation
IL-10	Mononuclear phagocytes, $CD4^+$ T cells, B cells, keratinocytes	Inhibits cytokine synthesis by macrophages, T cells, NK cells; downregulates MHC class II on macrophages; antagonises Th1 development; enhances B cell growth and antibody production; cofactor mast cell growth
IL-12	Mononuclear cells, B cells	Induces IFN-γ from T cells, NK cells; enhances T cell and NK cell cytotoxicity; growth factor for activated T cells; Th1 development
IL-13	T lymphocytes	B cell proliferation and immunoglobulin production; enhances IgE and IgG isotype switching; downregulates expression of macrophage inflammatory cytokines
IL-16	Many types; T cells, B cells, eosinophils, epithelial cells, mast cells	Chemoattractant for T cells, monocytes, eosinophils, growth factor for $CD4^+$ T cells
IL-18	Macrophages, keratinocytes	Augment T cell production of IFN-γ and TNF-α; related to IL-1 but uses different receptor
IFN-γ	T cells, NK cells	Increase expression MHC I and II; activates macrophage cytocidal activity; Th1 development; antagonises Th2
TNF-α	Mononuclear phagocytes, eosinophils T cells, NK cells, mast cells	Fever, enhances leucocyte endothelial interactions; cofactor for B-,nd T- cell proliferation; cytotoxic for some cell types
TGF-β	T cells, mononuclear phagocytes	Pleiotropic effects; generally and anti-inflammatory cytokine
GM-CSF	T cells, endothelial cells, monocuclear phagocytes, fibroblasts, mast cells	Growth of granulocyte macrophage precursors; enhances macrophage and granulocyte function
M-CSF	Monocytes, endothelial cells, fibroblast	Growth of mononuclear phagocytes; enhances macrophage function
G-CSF	Phagocytes, epithelial cells, fibroblasts	Promotes granulocyte production; enhances granulocyte function

To date in vitro work with *Leishmania* has focussed on using AM and freshly isolated LC (Moll, 1995). As DC can produce IL-12, are situated in the dermis for initial host contact with *L.major* PM and play a pivotal role in establishing cell mediated immunity they became an obvious target for the investigation of host response to infection with *L.major* PM.

1.4.6 Costimulatory molecule regulation

The mixed lymphocyte reaction (MLR) is used to test for histocompatibility by measuring the T cell proliferative response to foreign or allogeneic APC. $CD4^+$ T cells account for most of the cytokine production and proliferation in the MLR and DC are by the far the most potent APC in a MLR (Steinman and Witmer 1978). With the aid of transgenic mice, DC have been shown to be 100 to 300 times more efficient than any other APC in inducing a primary antigen-specific T cell response to soluble antigens in vitro (Macatonia, et al 1995). This is particularly relevant in the context of *L. major* where macrophages were considered to be the only cells capable of internalising parasites and the predominant APC in the primary immune response.

Antigen-loaded DC and antigen-specific T cells form aggregates that constitute a microenvironment optimal for the development of an immune response. An antigen-specific T cell immune response is initiated as a result of interaction between a T cell receptor (TCR) and antigen expressed on the surface of an APC in the context of MHC (Fig 1.8). TCR ligation initiates signal transduction but is not on its own sufficient to initiate an immune response under most circumstances. An additional

signal provided by ligation of APC costimulatory molecules with T cell receptors is essential for clonal T cell expansion and cytokine production. Without the second signal, ligation of the TCR alone can induce functional inactivation of T cells leading to a state of hyporesponsiveness or anergy (reviewed by Austyn 1998).

1.4.6.1 B7-1 (CD80), B7-2 (CD86)

The major costimulatory receptor on the surface of resting T cells is CD28 (Linsley and Ledbetter, 1993). CD28 has two distinct ligands on APC, B7-1 (CD80) and B7-2 (CD86). Both B7 molecules are members of the immunoglobulin gene superfamily and appear to be closely linked. In vivo, these two costimulatory ligands appear to differ in their ability to potentiate the differentiation of Th cells into Th1 or Th2 cells (reviewed by Thompson 1995). In several murine models, disease has been modulated by blocking co-stimulatory molecule ligation with concomitant reversal of Th cell phenotype. In the experimental murine model of allergic encephalomyelitis, simultaneous treatment with anti–B7-1 at the time of immunisation resulted in the generation of effector cells with a Th2 phenotype, abrogating disease. Treatment with anti–B7-2 resulted in the production of effector cells of a Th1 phenotype, resulting in increased disease severity (Kucheroo, et al 1995).

The B7 ligands also bind to CTLA4, a receptor on T cells closely related to CD28. In contrast to CD28, CTLA4 is not expressed on quiescent T cells but becomes detectable after TCR ligation and can be further augmented by CD28 costimulation. The role of CTLA4 in Th differentiation is not completely understood. Depending on circumstances, CTLA4 appears to be able to act as a costimulatory signal or as a

negative signal to down-regulate an immune response either by terminating a proliferative response or directly inducing apoptosis. CTLA4 binds B7-1 and B7-2 with higher avidity than CD28. A recombinant form of CTLA4, CTLA4Ig, has been used as a competitive inhibitor of CD28 activation. In vivo, CTLA4Ig treatment can suppress the ability to mount T cell-dependent antibody production as well as suppress the ability to mount a cell-mediated immune response against tissue grafts (reviewed by Thompson 1995).

The way in which B7 costimulatory molecules influence T cell differentiation is still unclear. It may be due to the timing of their expression in the immune response. B7-2 appears to be the predominant costimulatory molecule for activation of unprimed lymph node cells. In contrast B7-1 costimulation appears to be upregulated later during activation of an immune response and has been proposed to be the major costimulatory ligand present during persistent infections. B7-2 is rapidly induced and expressed at higher levels on dendritic cells compared to B7-1 (Inaba, et al 1994; Rattis, et al 1996). Furthermore, using antibody blocking experiments B7-2 was found to play a significantly greater role than B7-1 in the costimulatory function of dendritic cells.

The regulation of B7 expression is also under cytokine control. A range of cytokines have been explored with some conflicting findings, but consistently expression of both B7 ligands is induced by GM-CSF, IL-4 and down-regulated by IL-10. IFN-γ also down-regulates B7-1. These modulatory effects are maximal within the first few days of culture (Kawamura and Furue 1995). The involvement of these

costimulatory molecules are therefore of particular interest in the investigation of the early DC dependent development of cell mediated immunity to *Leishmania*.

1.4.6.2 CD 40-CD40L

CD40 and its ligand CD40L (CD 154), both members of the TNF receptor superfamily, were initially thought to be exclusively involved in the regulation of humoral immunity. CD40-CD40L plays a critical role in isotype switching of B cell gammaglobulin synthesis and a defect in CD40L causes the hyper-immunoglobulin M syndrome. CD40 is an integral membrane protein found on dendritic cells, B lymphocytes, follicular dendritic cells, macrophages and endothelial cells and interacts with CD40L expressed on activated CD4+ T cells, some CD8+ T cells and basophils/mast cells (reviewed by Banchereau, et al 1994). Using CD40 and CD40L deficient mice, researchers established that CD40L is also required for protective cell-mediated immunity to *Leishmania major* and *Leishmania amazonensis* (Campbell, et al 1996; Soong, et al 1996).

CD40-CD40L was found to be crucial for the synthesis of IL-12, NO and TNF–α by macrophages when challenged with *L.major* and *L.amazonensis* in-vivo. Furthermore, the CD40 ligation dependent IL-12 production by DC is also susceptible to downregulation by IL-4 and IL-10 (Koch, et al 1996). CD40L is also the most potent stimulus in upregulating the expression of other costimulatory molecules ICAM-1, CD80 and CD86 on DC (Cella, et al 1996). Via its regulatory function on the expression of adhesion molecules on endothelial cells, CD40L also effects the trafficking of effector T cells within the target organ (reviewed by Noelle

1996). CD40-CD40L has a clear role in the priming of naïve T cells via DC activation. The role of CD40-CD40L in the activation of the early immune response to *L.major* infection was investigated further in this work.

1.5 Dendritic cells

Dendritic cells are "professional" APC derived from bone marrow stem cells and distributed via the circulation to all tissues where they act as outposts of the immune system (reviewed by Knight and Stagg 1993). In the tissue they acquire and process antigen, then migrate by afferent lymph or blood stream to the paracortical T-dependent area of lymphoid organs where they initiate T-cell responses (Fig 1.10).

DC are a unique subset of APC. In addition to stimulating memory T cells, they are the only APC with the capacity to cluster and activate naive T helper cells. They are motile and have variable capacity for phagocytosis. Morphologically their name derives from the many elongated pseudopodial processes or dendrites which extend from the DC surface giving the cell a star like appearance (Fig 1.9). These dendritic veils provide an enormous cell surface area for antigen capture and presentation. The nucleus is generally horseshoe shaped and the cytoplasm has a paucity of organelles. They contain enzymes for degradation and processing of antigen, although little has been published in this area to date.

Precursor DC (DC_{pre}) derive from CD34+ bone marrow stem cells and circulate in the blood expressing MHC class II. They migrate to and become resident in non-

lymphoid tissues, such as epidermis (as LC) and other epithelia such as mucosa of the respiratory, urogenital and gastrointestinal tracts. They also become resident in the interstitial spaces of solid organs such as the heart and kidney but are absent in so called "immunologically privileged" sites, particularly the brain and testes. These resident DC are referred to as immature DC (DC_{imm}) equipped with the capability to take up and process antigen. Epidermal LC are probably the best studied DC_{imm} and are identified by expression of CD1a and the presence of Birbeck granules (cytoplasmic structures formed by double membrane joinings which resemble racquets on electron microscopy). They also express other molecules of the CD1 family, CD1c and CD1b which are important for antigen presentation (discussed below). Interstitial DC_{imm} lack the Birbeck granules and most do not have CD1 antigens (Bell, et al 1999).

Other surface molecules on DC_{imm} enable homing and adhesion to particular tissue sites (reviewed by Bell, et al 1999). These include DC_{imm} expression of CD49d β-integrin assisting passage through high endothelial venules into lymphoid organs, cutaneous lymphocyte antigen (CLA) interaction with E-selectin (CD62E) on activated endothelial cells in the skin and adhesion molecules ICAM-1, ICAM-2 and particularly E-cadherin on LC. The latter promotes adherence to surrounding keratinocytes which is down regulated when LC migrate out of the epidermis. CD44 is a heterogeneous multifunctional molecule involved in DC trafficking (Weiss, et al 1997). It is a receptor for the extracellular matrix component, hyaluronate, which is involved in lymphocyte homing and activation and is upregulated during migration of LC and DC to peripheral lymph nodes. Cytokines, particularly IL-1 and TNF-α,

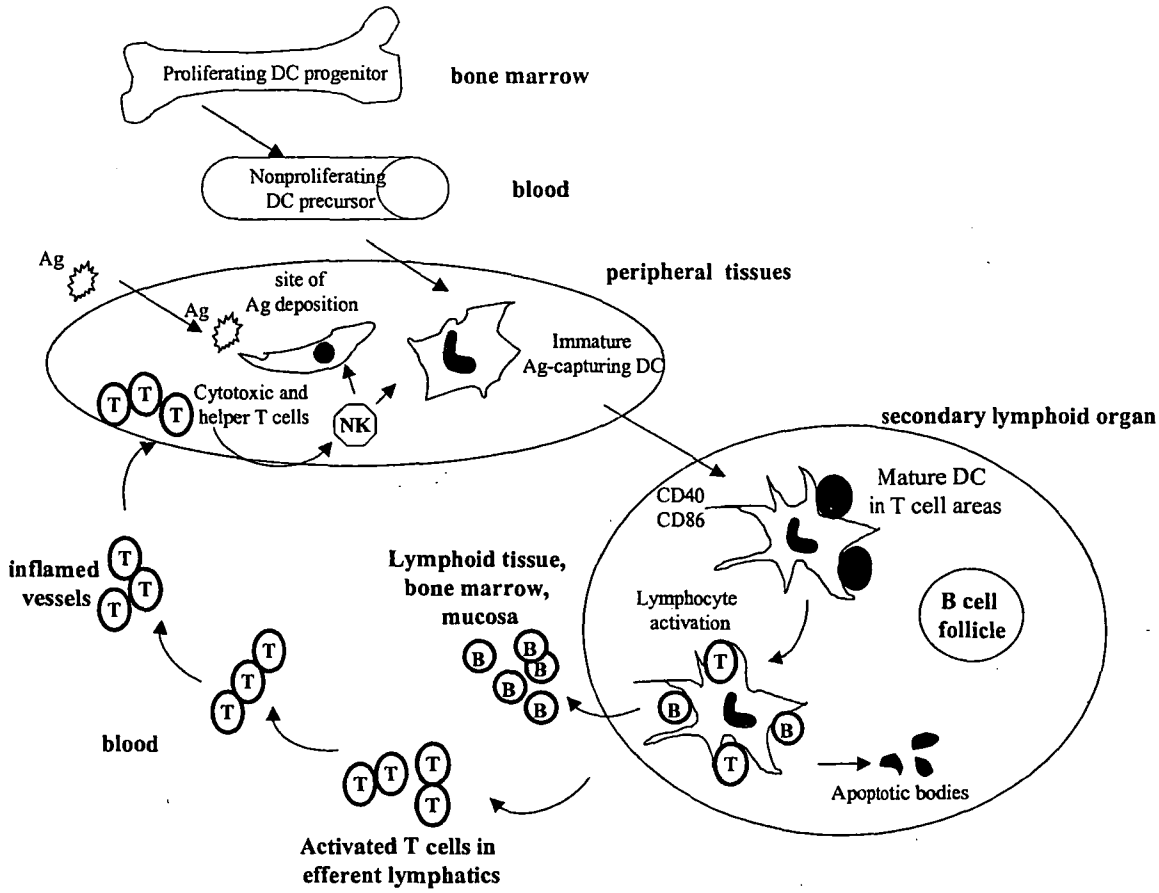
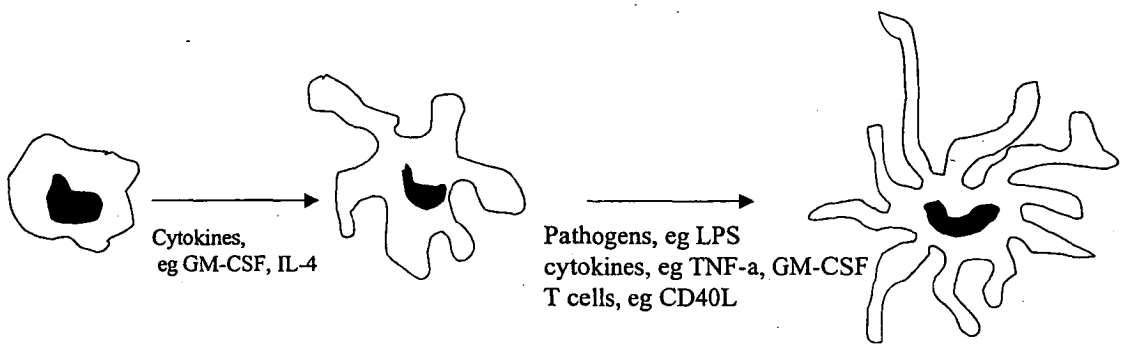


Fig 1.10. The life of a DC from progenitor to antigen capture and presentation to lymphocytes. Precursor DC derived from CD34+ bone marrow stem cells circulate to peripheral tissue where they are situated for antigen capture. The immature DC loaded with antigen mature and migrate to lymphoid organs where they express antigen peptides on their MHC molecules to rare circulating antigen-specific lymphocytes. These reactive T cells become activated and further induce terminal DC maturation which in turn promotes lymphocyte expansion and differentiation. Activated T cells selectively traverse inflamed epithelium and migrate back to the injured tissue. Helper T cells secrete lymphokines and cytotoxic T cells eventually lyse infected cells. Activated B cells differentiate into B lymphoblasts after contact with T cell and DC and migrate to areas where they mature into plasma cells and produce antibodies to neutralise the pathogen. (Adapted from Bell , et al 1999).

have also been shown to mobilise LC and assist in migration probably via downregulation of E-cadherin (Cumberbatch, et al 1997). DC can both produce and respond to chemokines, such as MIP-1α and MIP-1β and RANTES (Sozzani, et al 1995). DC_{imm} express chemokine receptors CCR1 (receptor for RANTES), CCR5 (receptor for MIP-1α, MIP-1β and RANTES and CCR6 (receptor for MIP-3α) which are downregulated with maturation and appear to play an important role in migration (Fig 1.11). Additionally LPS from bacteria can stimulate many cell types to produce these and other chemokines and cytokines which can modulate DC movement and maturation (Reis e Sousa, et al 1999).

The uptake of antigen may occur by one of several mechanisms. Macropinocytosis is a cytoskeleton-dependent type of fluid-phase endocytosis mediated by membrane ruffling and the formation of large vesicles (1-3μm) which is constitutively expressed in DC (Sallusto, et al 1995). LC use the high affinity IgE receptors (FcεRI) and most types of DC_{imm} use Fcγ receptors (CD32/FcγRII and CD64/FcγRI) for antigen capture. The mannose receptor involved in the internalisation of a variety of glycoproteins is expressed in high levels on DC. Unlike Fc receptors which are degraded with their antigen after internalisation, mannose receptors release their ligands at endosomal pH and are recycled. This enables uptake and accumulation of many ligands by a small number of receptors and probably plays an important role in phagocytosis of microbes (Svensson, et al 1997; Reis e Sousa, et al 1999). DEC-205, another endocytic receptor is discussed in more detail below. Finally engulfment of apoptotic bodies by DC_{imm} appears to be an efficient method of antigen capture for presentation, particularly in tumour and transplant immunology and in tolerance, which is beyond the scope of this review (Bell, et al 1999).



DC PRECURSOR
(DC_{pre})

IMMATURE DC
(DC_{imm})

high intracellular MHCII
endocytosis, FcR
high CCR1, CCR5, CCR6
low CCR7
low CD54, 58, 80, 86
low CD40, IL-12

MATURE DC
(DC_{mat})

high surface MHCII
low endocytosis, FcR
low CCR1, CCR5, CCR6
high CCR7
high CD54, 58, 80, 86
high CD40,IL-12

Fig 1.11. Schematic representation of surface molecule expression during DC maturation. When the DC precursors migrate into tissues to become resident DC_{imm} they are primed specifically for antigen uptake. After antigen capture DC_{imm} undergo maturation during migration to secondary lymphoid organs, with increasing expression of surface MHC II, chemokine receptors, costimulatory molecules and cytokine secreting capacity. (Adapted from Bell, et al 1999).

After antigen exposure, DC_{imm} migrate via afferent lymph as "veiled DC" to the draining lymph nodes or other secondary lymphoid organs such as tonsils and the white pulp of spleen (Fig 1.10), where they localise to the T cell areas as mature interdigitating DC (Hart and McKenzie, 1988; Stagg and Knight 1993). During migration the maturing DC undergo major changes in their phenotype. The mature DC (DC_{mat}) can be distinguished by upregulation of expression of critical epitopes such as costimulatory molecules, CD40, CD80 and CD86, MHC II, adhesion molecules ICAM-1 (CD54), LFA-3 (CD58), CD11a/c, actin-bundling protein p55 (fascin) and chemokine receptor CCR7 (Fig 1.11). Concomitantly the Fc receptor expression decreases along with most of the other chemokine receptors (Bell, et al 1999).

MHC class II loading of antigen is critical for activation of CD4+ T cells. In DC_{imm} most of the intracellular class II MHC is found in late endocytic structures called MIICs (MHC class II compartments). As the DC mature they accumulate class II antigen in nonlysosomal vesicles (CIIV) and finally as DC_{mat} peptide-class II MHC complexes are found on the plasma membrane for long periods of time, allowing the selection of rare antigen-specific T cells (Pierre, et al 1997).

1.5.1 DC development

The development of culture techniques using growth factors and cytokines, in the 1990s, enabled in vitro generation of large numbers of DC from haemopoetic origin. Subsequently it has been recognised that distinct developmental pathways give rise to several discrete subsets of DC (reviewed by Austyn 1998; Bell, et al 1999). They can

be divided into LC, myeloid DC, monocyte-derived DC and lymphoid DC. The first three appear to reside transiently in nonlymphoid tissues before migrating to secondary lymphoid tissues for the induction of immunity against foreign antigens. In contrast, lymphoid DC probably enter lymphoid tissues directly from the blood and play a role in the induction or maintenance of tolerance to self antigens. In this aspect, human DC have been the most studied.

Myeloid DC and LC can be derived from $CD34^+$ progenitors from cord blood or bone marrow by culture with TNF combined with either IL-3 or GM-CSF (Caux, et al, 1992). These cultured $CD34^+$ develop along two independent pathways, defined by mutually exclusive expression of CD1a and CD14 at an intermediate stage. Both populations eventually mature into DC but of 2 different types: $CD1a^+CD14^-$ intermediates give rise to $CD1a^+CD14^-$ LC (Birbeck granules, Lag^+, E-cadherin$^-$) while the $CD1a^-CD14^+$ cells become interstitial (dermal) DC which are $CD1a^+CD14^-$ (lack Birbeck granules, Lag^-, E-cadherin$^-$, $CD2^+$, $CD9^+$, $CD68^+$, Factor $XIIIa^+$). The two DC types are also characterised by specific activities (Caux, et al 1997). Interstitial DC have a potent and long lasting antigen uptake activity compared to LC which is mediated by mannose receptors. They are also uniquely able to induce naïve B cells to differentiate into IgM-secreting cells in response to CD40 ligation and IL-2. The addition of TGF-β1 to similar cultures promotes LC development and inclusion of c-kit ligand (stem cell factor, SCF) increases yield (reviewed by Austyn JM 1998). DC can also be cultured from peripheral blood $CD14^+CD1a^-$ mononuclear cells but differ from $CD34^+$ derived DC in their requirement for GM-CSF combined with either IL-4 (monocyte suppressor) or IL-13. These DC are not terminally differentiated and resemble DC_{imm} (Bell et al 1999).

In mice a similar situation occurs where bone marrow $CD34^+$ progenitors can be driven along a DC differentiation pathway in the presence of GM-CSF. The addition of LPS or TNF-α induces further maturation. Flk-2/Flt-3 ligand increases the yield of DC in this culture system (reviewed by Austyn 1998).

The fourth type of DC, lymphoid DC, have been described in both mice and humans. Altogether the evidence suggests that these bone marrow derived DC progenitors seed the thymus and generate DC which become involved in the induction of tolerance. Lymphoid DC progenitors also become parafollicular, interdigitating DC in the T cell-rich areas of secondary lymphoid tissues directly from the blood. In the latter, distinct subsets occur. $CD8a^-DEC205^-CD11b^+$ DC can be isolated from the marginal zones of the white pulp of the spleen and $CD8a^-DEC205^+CD11b^-$ DC from lymph nodes (Vremec and Shortman 1997; Steinman, et al 1997). The origin of these DC in unclear. They may originate from non-lymphoid tissues and enter through the marginal zone of the spleen and the subcapsular space of lymph nodes ultimately homing to T-cell areas for cognate T cell interaction. An exciting possibility is that DC originating from different sites may induce different patterns of T cell cytokine production, specifically Th1 from spleen DC and Th2 from Peyers patches (Everson, et al 1996).

The pivotal role of DC is becoming increasingly apparent in many diseases and conditions. These range from autoimmune diseases to transplant and tumour immunology and infectious diseases. The former areas are reviewed elsewhere (Bell, et al 1999; Austyn 1998). The role of DC in leishmaniasis is further considered.

1.5.2 Role of DC in Leishmaniasis

Leishmania is included in the growing list of intracellular pathogens, HIV, *Influenzae,* herpesviruses, *Chlamydia*, *Listeria*, *Mycobacteria* and *Candida,* in which DC have been found to be critical in regulating the host's immune response (reviewed by Bell et al 1999). Heidrun Moll's group first observed that murine epidermal LC were potent stimulators of antigen-specific T cell responses to *L.major* (Will,et al 1992). They then demonstrated that murine LC could take up whole *L.major* parasites in vitro (Blank, et al 1993). Internalised parasite numbers were small (average of two per cell) and uptake appeared to be via the complement receptor CR3 on LC. This observation underlined the purpose of LC as specialised APC rather than non-specific scavenging cells. These LC also appeared to harbour persistent parasites even after migration to regional lymph nodes where they stimulated antigen-specific T cell responses (Moll H, et al 1995). However, a recognised limitation of their study was the restricted capacity of the LC to internalise *L.major* only in the AM form of the parasite. It is the PM form of the parasite which is transmitted by sandfly bite and will be encountered in the critical early stages of the host immune response. The interaction of PM with DC had not previously been examined prior to this thesis.

As previously discussed, macrophages are paralysed by PM infection and not capable of producing IL-12. Recent evidence from in vivo experiments suggests that DC rather than macrophages may be the major source of IL-12 during the early stages of parasite infection (Reis E Sousa, et al 1997). The question therefore was whether DC

exposed to *L.major* PM were the source of IL-12 and whether they could activate naïve T cells to initiate primary immune responses in vitro.

1.5.3 CD1 presentation of non-peptide antigen

DC express CD1 molecules. These comprise a more recently described important family of non-MHC antigen presenting molecules. The CD1 antigens are nonpolymorphic cell surface polypeptides, non-covalently associated with β2-microglobulin and bear some structural similarity to MHC molecules. They play an important role in presenting nonpeptide, lipid-containing microbial antigens to T cells (Porcelli, et al 1992). They have been shown to be involved in host immune responses to *Mycobacterium tuberculosis* and *M.leprae.* DC bearing CD1 at sites of *M. leprae* infection portend a better prognosis (Sieling, et al 1999). Downregulation of $CD1^+$ on DC infected with *M. tuberculosis* while maintaining MHC molecule expression has been suggested as a mechanism of an evolutionary evasive adaptive response by the pathogen (Stenger, et al 1998).

CD1 molecules have the potential to play an important role in the early immune response to *L.major* infection, particularly as several of the immunogenic epitopes, such as LPG are nonpeptides and could be presented by DC to T cells in the context of CD1. This area had not been previously investigated and preliminary work was undertaken to further purify nonpeptide antigen from *Leishmania* cultures and discussed in more detail below.

1.5.4 DC surface membrane receptor

The role of the mannose-fucose receptor in macrophage uptake of *Leishmania* PM is well established (Mosser, et al 1987). As described above, recent evidence suggests that mannose receptors on the cell surface are important in acquisition of antigen by DC. Specifically, DEC-205, an integral membrane protein homologous to the macrophage mannose receptor was found to be expressed at high levels on murine DC and was associated with antigen presentation (Jiang, et al 1995). DEC-205 binds carbohydrates and mediates rapid endocytosis by internalising coated pits and vesicles and is delivered to a multivesicular endosomal compartment that resembles the MHC class II-containing vesicles. Furthermore, antigens targeted to these receptors are presented by MHC class II 100-10 000 fold more efficiently than untargeted proteins (Tan MC, et al 1997). DEC-205 is found on the lymphoid subset of DC as outlined above. The monoclonal antibody, NLDC-145, recognises this surface membrane protein and is used as a specific DC marker (Kraal, et al 1986). The possibility of involvement of this DEC-205 as a DC receptor involved in the uptake and processing of *Leishmania* was investigated.

1.6 Vaccine development

Immunisation against cutaneous leishmaniasis has been practised traditionally in Middle Eastern countries for a long time. Mothers expose their babies' buttocks to sandfly bites to prevent infection occurring on exposed body parts later in childhood, where disfiguring scarring may lead to social discrimination, particulaly in women. This practice and the innoculation of live PM in Israel and former Soviet Union has

been effective in preventing naturally acquired disease but is hampered by parasite persistence, delayed healing of resulting lesions and complications with secondary bacterial infection. Clinical trials of killed parasite have had only limited success. A killed *Leishmania* PM vaccine without adjuvant was used on troops in Brazil but gave limited protection (reviewed by Pearson, et al 2000). A large randomised study in Iranian schoolchildren comparing vaccination with clinical-grade preparation of autoclave-killed *L.major* (ALM) + BCG was only marginally more efficaceous than BCG alone (Sharifi I, et al 1998). The search for an effective, non-disease producing vaccine was aided by immunological advances in understanding the mechanisms of an effective primary immune response discussed above.

The murine model of *L.major* infection has established that effective primary immunity to *L.major* infection results from the development of a Th1 response characterised by the IL-12 dependent production of IFN-γ from MHC class II-restricted $CD4^+$ T cells and from NK cells, as reviewed above. These observations form the basis of the current development of vaccines against leishmaniasis. Most vaccines used in humans induce neutralising antibodies to provide long term protection. For certain intracellular pathogens, including *Leishmania*, where cellular immune responses are necessary for protection, the development of suitable vaccines which are able to induce durable responses is hampered by the lack of understanding of the factors required for this. Effective vaccination against *L.major* in susceptible mice strains was achieved using recombinant IL-12 (rIL-12) with soluble leishmanial antigen (SLA) and subsequently with a recombinant leishmanial protein (LACK) (Afonso, et al 1994; Mougneau, et al 1995). Investigations have been extended to primate studies, but protective immunity was not long lasting. Adjuvants such as

alum (aluminium hydroxide gel) when used in addition to rIL-12 with heat-killed *Leishmania amazonensis* in rhesus macaques provided protective immunity to rechallenge at four weeks (Kenney, et al 1999).

Advances have been made using DNA vaccination stategies which induce both MHC class I- and class II-restricted responses (reviewed by Gurunathan, et al 1998). The enhanced durability of DNA vaccines is probably attributible to $CD8^+$ T cell production of IFN-γ. Vaccinating susceptible mice with plasmid DNA encoding the LACK antigen without additional adjuvant has been shown to confer protective immunity for over 12 weeks. Similar results were obtained using a clinical-grade preparation of ALM plus IL-12 DNA. Corresponding reduction in IL-4 producing T cells was also observed when the mice were rechallenged at 12 weeks. These vaccines also substantially reduced the parasite load in resistant strains of mice after infection. There appears to be a requirement for persistence of IL-12 for the continual induction of IFN-γ production and to prevent apoptosis in Th1 cells. Other researchers have confirmed that immunostimulatory DNA sequences (CpG-containing oligodeoxynucleotides) co-inoculated with ALM drive the Th1 effector response via IL-12 and IFN-γ-dependent mechanisms (Walker, et al 1999).

Potential candidates for a subunit vaccine include recombinant *Leishmania* proteins such as LeIF (Skeiky, et al 1995), synthetic immunogenic epitopes from the surface glycoprotein gp63 (PT3) and cysteine proteinases which elicit Th1 responses in susceptible mice, and PSA (PM surface antigen-2) which also induce in vitro proliferative responses from peripheral blood mononuclear cells of human leishmaniasis patients (Kemp, et al 1998). Of particular interest is the possibility of

using DC to enhance the potency and longevity of a protective immune response to *Leishmania* infection. A single intravenous application of LC pulsed with *L.major* antigen in vitro provided protection in susceptible BALB/c mice against subsequent challenges with *L.major* parasites (Flohe, et al 1998). Genetic engineering of human DC to secrete functional IFN-γ and IL-12 as part of immunotherapy for leishmaniasis and other infections is another new and exciting area under investigaton at present (Ahuja, et al 1998).

1.7 Specific Aims

The aim of this thesis was to investigate whether DC play an influential role in naïve T cell differentiation and therefore host susceptibility and resistance in the murine model of leishmaniasis. Using an in vitro murine model of *L.major* infection, the following specific aims were the focus of this research:

- To determine if DC internalised PM.
- To determine if DC exposed PM were capable of initiating naïve T cell proliferation.
- To determine if PM-exposed DC influenced naïve T cell cytokine production.
- To determine if PM-exposed DC produced IL-12
- To examine if resistant and susceptible strains differed in any of the above.
- To determine if specific DC endocytic receptor, DEC-205, was involved in *L.major* uptake.
- To determine if a non-peptide excreted factor from *L.major* PM could be presented by DC to stimulate primary T cell responses.

An in vitro model was used to minimise as much as possible the complex intercellular communication which occurs in vivo so that the effect of isolated cytokines and specific intercellular reactions could be analysed under controlled conditions. To simulate as closely as possible the initiation of infection, the infectious PM stage of the parasite was used in cellular cultures with DC, macrophages and T cells of naïve mice of both susceptible and resistant phenotype.

In vitro derivation of DC from stem cell cultures with cytokine enriched media, as discussed above was not considered ideal for this work. Although significantly greater quantities of DC would be available for experimentation using this method, these DC are already cytokine influenced by the inherent nature of their generation process and may not reflect the "naïve" situation. On the other hand LC had already been used in murine studies and did not appear to be involved in uptake of the PM form of *L.major*. Immature DC were required and splenic DC were for technical purposes considered to be the most suitable. The different lineages of DC have been more clearly defined subsequent to this work.

In summary, an in vitro murine model was developed to observe DC interaction with the infectious PM stage of *Leishmania* and the subsequent influence of *Leishmania*-exposed DC on the pattern of T cell development. Whether DC harbour the parasite or predominantly take up *Leishmania* for rapid processing and presentation of the parasite antigens to T cells was explored. The role of a recently characterised specific DC membrane protein as a potential candidate receptor for the uptake of *Leishmania* into DC was also investigated. *Leishmania major* PM cultures were set up in the laboratory and the optimum conditions determined for harvesting the most

infectious PM stage. Immature dendritic cells were isolated from spleens of young adult naive mice reared in pathogen free conditions using an established method of overnight adherence step and further purification. Firstly interaction of parasite with DC was assessed from parasite: DC co-cultures using light microscopy, fluorescence and electron microscopy and fluorescent activated cell sorting (FACS) analysis. The role of the DC receptor, DEC-205, by *Leishmania* was assessed with blocking antibody studies. DC production of IL-12 was assessed from the DC:*Leishmania* co-cultures. *Leishmania*-exposed DC were then used in T cell proliferation assays to investigate primary T cell proliferation and T cell cytokine production, particularly IFN-γ and IL-4.

In Chapter 2 the methods and materials used in this research are outlined while the results are presented in Chapter 3. In Chapter 4 the results are discussed. A supplementary study funded by a WHO Director's Initiative Grant on which I was a co-applicant, to investigate a novel therapy for the treatment of cutaneous leishmaniasis refractory to routine therapy in Aleppo, Syria is described in Chapter 5. Finally, in Chapter 6, the findings and the implications that these results have for future research is discussed and final conclusions are drawn.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Chemicals, reagents and media

Most reagents, chemicals and media were provided ready for use in experiments. Where further preparation or modification occurred in the laboratory, details are outlined below.

Complete medium

Dutch modification of RPMI 1640 (Gibco, Paisley, UK) was supplemented with 10% vol/vol fetal calf serum (FCS; Gibco, Grand Island, NY), 100 IU/ml penicillin, 100μg/ml L-glutamine and 5×10^{-5} M 2-Mercaptoethanol for culture of all mouse derived cells. For brevity it is referred to as complete medium (CM).

Metrizamide

Aliquots of 14.5g metrizamide (Nygaard, Oslo, Norway) were dissolved in 90mls Dutch modification of RPMI 1640 supplemented with 10% vol/vol FCS, 100 IU/ml penicillin and 100μg/ml L-glutamine such that a concentration of 13.7% w/v metrizamide was achieved. The solution was passed through a 0.45um filter to exclude bacterial contamination. The metrizamide solution was then used in 2ml aliquots for centrifugation of spleen cells to isolate low density cells, as described below.

Buffers

FACS buffer was made by supplementing 500mls of sterile phosphate buffered saline (PBS) with 10mls of FCS (2%), 0.1g of sodium azide (0.02%) and 0.19g

EDTA(1mM), shaking to dissolve. It was kept refrigerated at 4°C. Mini-Macs buffer was made by supplementing 500mls of PBS with 5 mls EDTA and 0.5% bovine serum albumin.

2.2 *Leishmania* Parasites

Leishmania major is the species of *Leishmania* for which clear cut genetic predisposition to disease resistance and susceptibility has been best characterised and is therefore the species most frequently used in the experimental model of leishmaniasis. As this work seeks to answer questions central to disease outcomes and to remain consistent with the bulk of current research, *L.major* was selected for this model.

Leishmania major strain JISH 118, originating in Saudi Arabia and passaged through BALB/c mice, was kindly provided by Dr Simon Croft, London School of Hygiene and Tropical Medicine. The PM were propagated in vitro at 25^{0}C in Dutch modification of RPMI 1640 supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2mM glutamine and 10% vol/vol. During the first week of passage, the PM undergo an exponential log phase of growth and characteristically have slender, elongated forms. They reach stationary phase by day 5 -7 at a density of approximately $3.7x10^{7}$/ml (Fig 2.1). At this point the liquid culture medium is exhausted and acidification helps to induce transformation to infectious metacyclic PM (Sacks and Perkins 1984). The metacyclic PM are shorter and highly motile with a long but tightly coiled, extremely active flagellum protruding from a bulbous apex (Fig 2.2). They comprise up to 85-90% stationary phase PM. Due to the

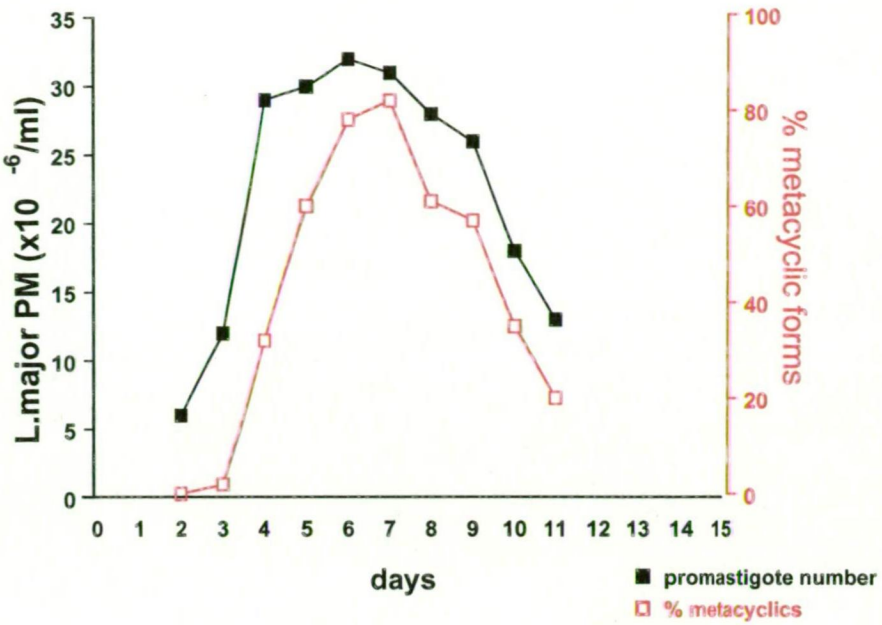


Fig. 2.1 Growth curve of *L.major* PM in liquid culture medium at 25°C. Stationary phase is reached between day 4 and 7 when PM density reaches $3x10^7$/ml.

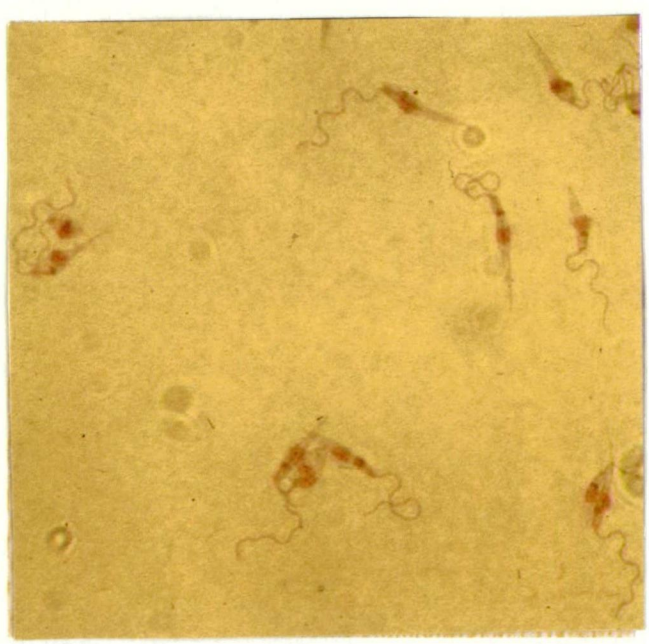


Fig 2.2 Stationary phase metacyclic *L.major* PM (10-20 μm) in liquid culture at day 5 with characteristic morphology of extensively coiled, highly motile flagella protruding from bulbous apical end of an elongated body containing a round nucleus and rod-shaped kinetoplast.

high density of PM, for co-culture experiments PM were added directly from the stationary phase cultures in small microlitre volumes to the DCs, such that the PM:DC ratio was approximately 5:1, as described further below.

To maintain a supply of parasites, PM from the 1st sub-passage were stored at -70^{0}C in 45% FCS and 10% dimethylsulphoxide (DMSO). When needed, aliquots were thawed for further passaging by rapid warming at 37^{0}C in Dutch modification of RPMI medium supplemented with glutamine, penicillin and streptomycin with 20% FCS and washed to remove residual DMSO. A maximum of five sub-passages were used for in vitro experiments to ensure optimal parasite integrity and infectivity.

Leishmania are routinely cultured in parasite specific medium, such as Schneider's medium, which vary in composition to RPMI modified Dutch medium, a more general cell culture medium which was used for in vitro cell culture in this work. To ensure that using the latter had no detrimental effect on parasite development, the two culture mediums were compared measuring PM concentration at day 5, 7 and 12 of the first passage after receiving the parasites in Schneider's medium from the London School of Hygiene & Tropical Medicine (Table 2.1).

Parasite-free culture supernatant was obtained from the stationary phase cultures by centrifugation through a 0.22μm filter at 600g for 5min.

Day of passage	Schneider's + 10% FCS (PM x 10^7/ml)	Complete medium (PM x 10^7/ml)
5	0.96	2.83
7	2.4	3.6
12	4.7	4.4

Table 2.1 Comparison of two culture mediums, Schneider's and complete medium demonstrating that complete medium is as supportive as Schneider's medium for *L.major* PM development to stationary phase ($3.7x10^7$/ml) by day 7.

2.3 Animals

Male C3H, C57/BL10 and BALB/c mice, 8 - 12 weeks old, were bred and reared in the specific pathogen free unit at Northwick Park Institute for Medical Research, Harrow, Great Britain. They were sacrificed by cervical dislocation. The C3H and C57/BL10 mice were the resistant phenotype and BALB/c, the strain susceptible to disease as described in chapter 1.

2.4 Cell isolation

2.4.1 Dendritic cells

The number of DC circulating and resident in tissue are very small. To obtain sufficient cells for in vitro experimentation mouse spleen was used.

DC were isolated and purified using a standard method in the laboratory. Single-cell suspensions were prepared by pressing aseptically removed mouse spleen through a gauze cell strainer (40μm, Falcon) and washing in complete medium. The cell suspensions were incubated overnight in 25cm^2 Falcon tissue culture flasks at 37^{0}C, 5% CO2 at a concentration of approximately 4 x 10^6/ml. After incubation, non-adherent cells were aspirated from the cultures and gently layered over 2ml of 13.7% w/v metrizamide (Nygaard, Oslo, Norway). Centrifugation at 600g for 10 minutes separated the denser lymphocytes from the low density cells (LDC) which appear as a milky white interface at the top of the metrizamide layer. The LDC comprise about 80-90% DC (Knight SC, et al 1983). LDC

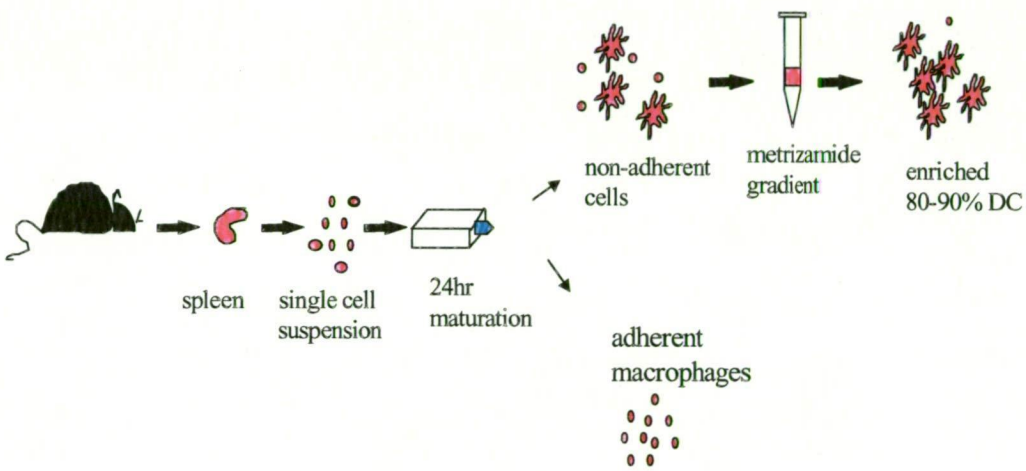


Fig 2.3 Isolation of DC and macrophages from mouse spleen

enriched for DC were aspirated from the interface, washed and counted (Fig 2.3). This method produced approximately 0.5 – 1 x 10^6 DC per spleen.

With overnight incubation, the DC mature upregulating Class II and costimulatory molecules on their surface. For parasite uptake experiments, the period of incubation was varied to allow for collection of populations of DC of varying maturity. For mature DC, 17 - 24 hours incubation on plastic was required

2.4.1.2 Further DC purification

To enable positive selection of DC from LDC populations, a specific mouse DC antibody, NLDC-145 (Kraal GM, et al 1986) was kindly provided by Dr Kraal, Amsterdam. NLDC-145 was derived from supernatants of an NLDC-145 cell hybridoma line. The antibody was biotinylated using a biotinylation kit (Amersham, UK) with a biotin-streptavidin immunodetection system. Specifically, concentrated NLDC-145 supernatants were dialysed overnight into a sodium phosphate buffer with pH 7.2 using a slide-A-lyzer kit. Biotinylation reagent (biotinamide-caproic acid) was added to the supernatant at a ratio of approximately 50ul per 1mg of protein (as determined by UV absorbance at 280mm) and incubated at room temperature for one hour with constant agitation. The sephadex column for separating biotinylated protein from unbound biotin was equilibrated with 25mls of PBS containing 0.1% BSA. The 1ml NLDC supernatant sample was applied to the column and fractions of 1ml collected by

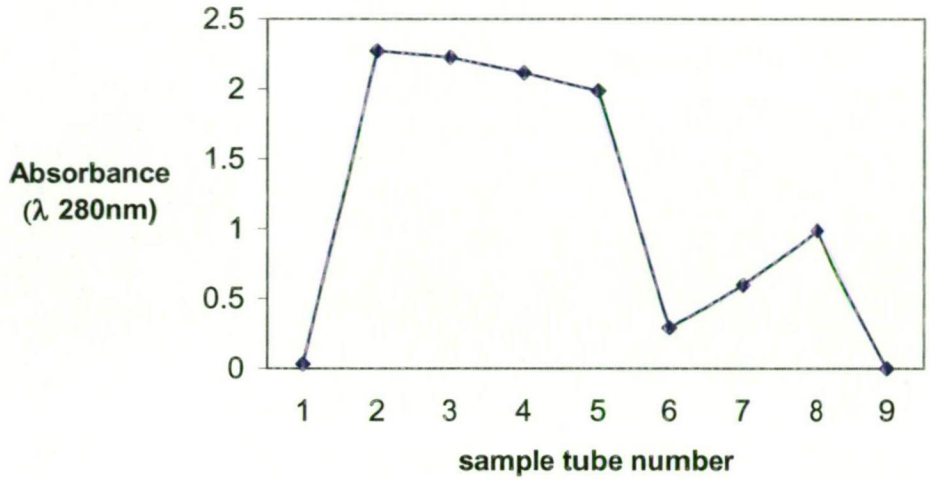


Fig 2.4 Biotinylation of NLDC-145. Nine 1ml samples of NLDC-b in PBS were eluted from the sephadex column and protein content estimated using absorbance spectrophotometry.

elution with 9mls of PBS + 0.1% BSA solution. The 9 samples were tested for protein content using a spectrophotometer zeroed at wavelength of 280nm, the absorbance wavelength for IgG. Samples 2 to 5 had the highest protein content, as expected, and these were combined to proved the stock biotinylated NLDC-145 (NLDC-b) for this research (Fig 2.4). Assuming the majority of the protein was IgG and using 1.4 as the extraction coefficient of IgG, the concentration of NLDC-b was calculated to be approximately 1.57 mg/ml or 10 nmoles/ml.

The efficacy of the biotinylated NLDC was assessed. Positive labelling of the DC population was confirmed using 10^5 LDC per 20ul volume exposed first to rabbit serum for 15 minutes, then biotinylated antibody or isotype for 20 minutes and washed twice. An avidin-FITC layer was applied for 20 minutes followed by 2 washes and acquisition of data with the FACScan. A dose titration curve was obtained using 2ul, 5ul and 10ul aliquots of NLDC-b. Equal specific labelling occurred at 5ul and 10ul aliquots (Fig 2.5) and 5ul aliquots of NLDC-b were therefore used for further experiments.

The NLDC-b was used in experiments which required further purification of DC from the LDC population. Highly purified DC were obtained by positive selection using immunomagnetic Mini-Macs columns (Miltenyi, Germany) and NLDC-b. To enable maximal availability of magnetic binding surface area on the column, non-viable cells from the non-adherent cell layer (which had a tendency to stick to the column) were removed by centrifuging the sample over Lympholyte M gradient for 20 minutes at 2400 rpm. Cells from the interface were collected and

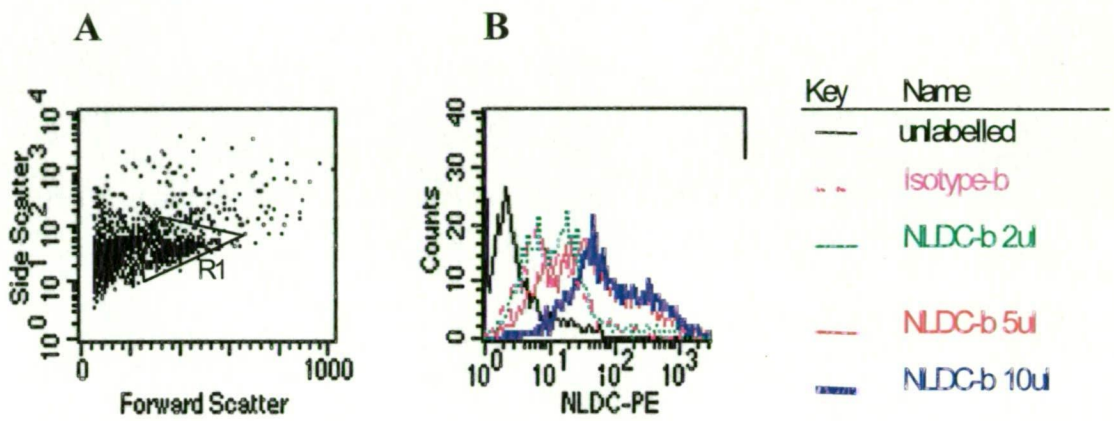


Fig 2.5 Efficacy of the NLDC-b was established and dose titration ascertained that 5µl was sufficient for optimal DC labelling. A. Dot plot showing the DC region (R1) which labelled positively for NLDC-b. B. Histogram of the level of labelling with increasing concentration of NLDC-b and isotype control.

layered over metrizamide to isolate the LDC fraction as described above. The LDC were then resuspended in Mini MACS buffer and incubated on ice initially with rabbit serum for 15 minutes to reduce non-specific binding. Biotinylated NLDC-145 was then added for 30 minutes at 40ul per 500 000 cells. Following two washes, avidin-microbeads were added and the cells incubated at 4^0 C for 15 minutes before two further washes. The cells were then passed through a Mini MACS column to which the iron containing microbeads with attached NLDC-b-labelled DC adhered. Thus positively selected cells were washed from the column after removal of the column from the magnet. The NLDC+ve DC, comprising >95% of LDC, were then available for culture after resuspension in complete medium (Fig 2.6).

There were potential limitations to using biotinylated NLDC-145 as the only method of purifying DC. Firstly, NLDC-145 labelling may occur in activated macrophage populations (Kaye 1987) and the aim was to ensure exclusion of macrophages from the cell population analysed. NLDC-145 appears to label splenic DC of lymphoid origin and therefore may exclude potentially important DC of non-lymphoid origin from analysis. CD11c is another marker which may be used to identify DC. $CD11c^+$ DC, mostly found within germinal centres, are relatively immature DC which quickly mature in vitro and are potent T cell stimulators (Grouard, et al 1996). Therefore in other experiments positive selection of DC with a mAb to CD11c bound to microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) was also performed for IL-12 studies. Purity was confirmed by FACS analysis following labelling with CD11c (clone HL3, PharMingen).

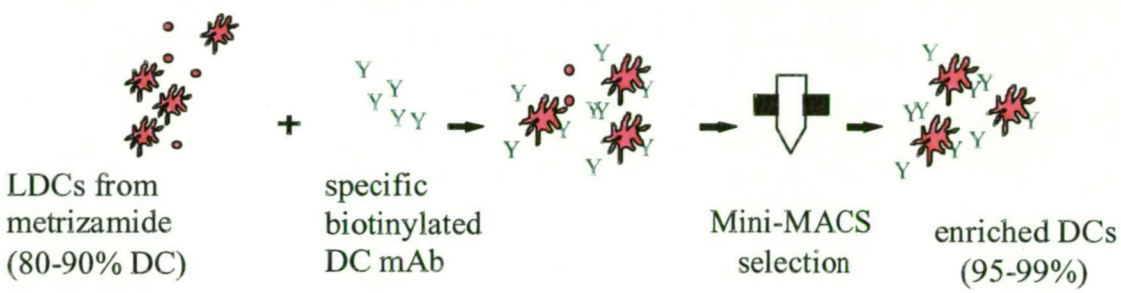


Fig 2.6 Further purification of splenic LDC using positive selection of DC from the LDC derived over a metrizamide gradient with biotinylated specific DC mAb (either NLDC-145 or $CD11c^{+}$) and Mini-MACS column. DC comprised >95% of selected population.

2.4.2 Macrophages

Macrophages were used as positive controls in experiments analysing DC uptake of parasites. Macrophages were obtained following overnight culture of splenic cell suspensions prepared as above. Adherent cells were collected by dissociation from the tissue culture flasks in complete medium after refrigeration at 4^0C for 15 minutes. Viability was determined by trypan blue dye exclusion and functional integrity was confirmed by their ability to phagocytose zymosan particles soaked in nitro blue tetrazolium (NBT, Sigma) and to subsequently reduce the NBT to blue-black formazan.

2.4.3 Lymph node cells

To assess the effect of *Leishmania*-exposed DC on autologous lymphocyte proliferation, lymph nodes were excised aseptically from the same mice that the spleens were taken. Single cell suspensions were obtained by pressing axillary, inguinal and brachial lymph nodes through a gauze cell strainer (40μm, Falcon). LNCs were washed twice in 8mls of CM for 10 minutes at 2000rpm and the LN pellet resuspended in 0.5-1.0ml CM prior to use. In later experiments, lymphocytes were further purified to T lymphocytes, by extraction of B lymphocytes over nylon wool columns. Specifically, using a sterile technique, 0.6g of boiled and dried nylon wool was teased out to increase the surface area and autoclaved. It was then placed in a test tube with a 3 way tap and stop cock turned off. The wool was then soaked with 10mls of CM and warmed to 37°C. The LNC collected above were resuspended in 2mls of CM. The column of nylon

wool was compressed to 6-7 mls by opening the stopcock and draining excess CM. The LNC suspension was added dropwise to the column and an additional 1ml of CM added to cover the top of the column. The column was incubated at 37^0C for a further 45 minutes facilitating adhesion of B cells to the nylon wool. The non-adherent T cells were then displaced by adding 10mls of CM dropwise to the column and collecting the effluent below. The purified T cells were then washed and resuspended in 1ml of CM for lymphocyte proliferation assays. Flow cytometric analysis confirmed moderate depletion of CD19 positive lymphocytes using this method (Fig 2.7).[2]

2.5 Quantification of DC infection

The initial phase of the research explored the possibility of spleen derived DC internalising *L.major* PM. Light microscopy was employed in the first instance. DC were identified by their typical morphology. Specifically, they are large mononuclear cells with elongated processes, a horseshoe shaped nucleus and paucity of organelles. When uptake of parasites was observed using this technique, further confirmation of the identification of the cells as DC and quantification of internalised parasites was performed with uptake studies using fluorescence and electron microscopy.

[2]Elimination of B cells removes the possibility of B cells acting as APC for the lymphocyte proliferation assays. Using this method, however, also increases the CD8:CD4 ratio to approximately 50:50 which must be borne in mind when analysing the cytokine assays from the in vitro cultures.

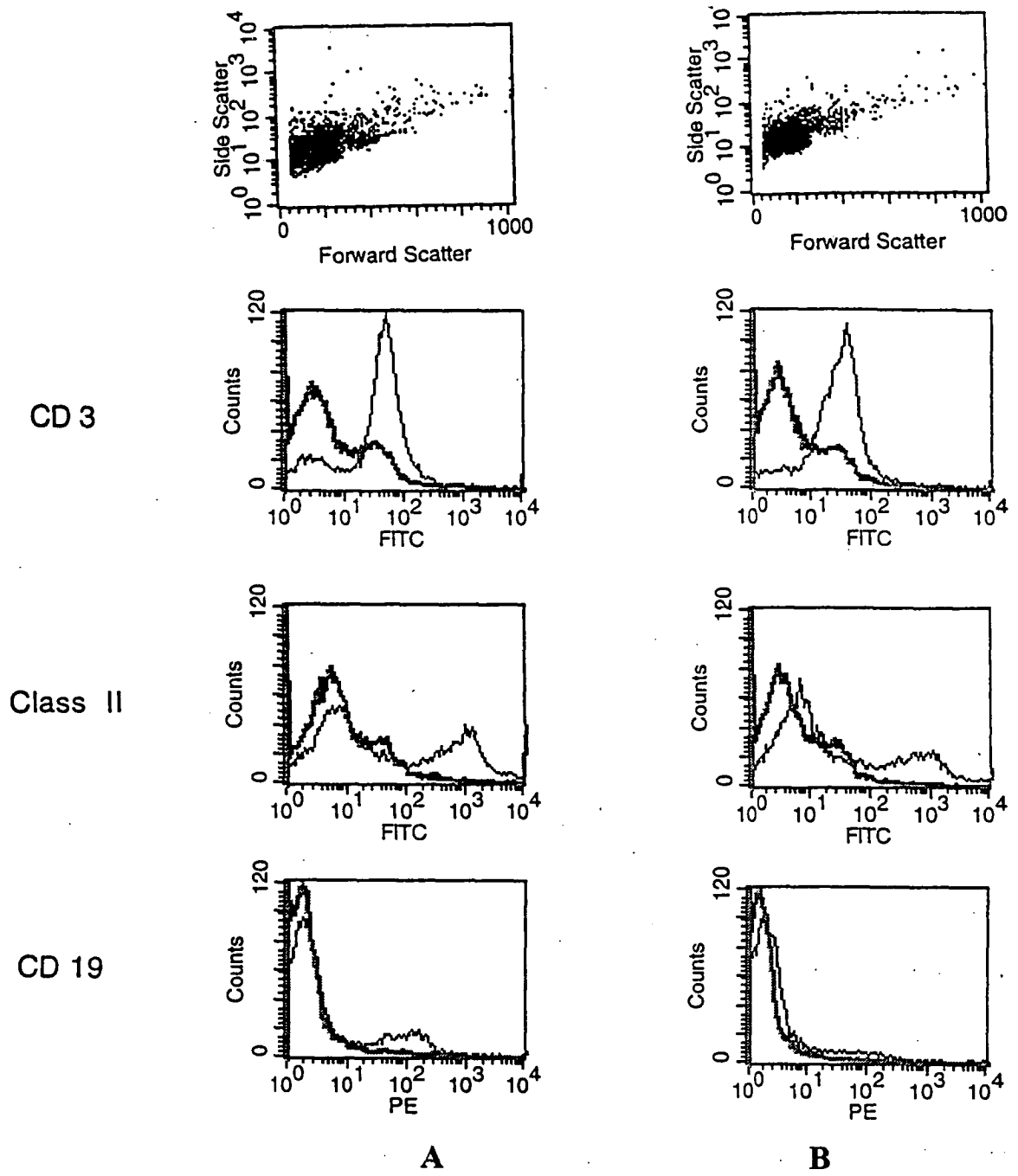


Fig 2.7 Freshly isolated lymphocytes (A) were enriched for T cells (B) after passing over nylon wool columns as demonstrated by flow cytometry. A high level of Class II and CD3 is maintained while the level of CD19, a B cell marker, has been reduced by the columns. The bold lines represent isotype controls.

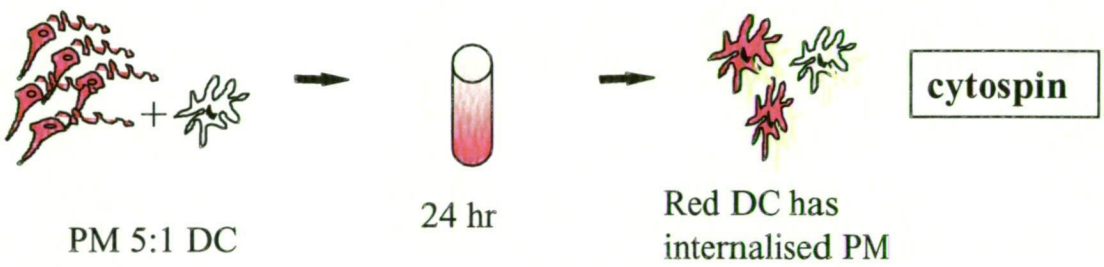


Fig 2.8 Preparation of DC:PM co-cultures for light microscopy. PM are incubated with DC in a ratio of approximately 5:1.

2.5.1 Light microscopy

DC containing intact intracellular parasites were quantified by light microscopy of DC:PM co-culture samples which had been prepared on microscope slides by cytocentrifugation at 600g for 5 mins, fixed with methanol and stained with Giemsa (Fig 2.8). DC were suspended at approximately 5x 10^6 cells/ml for use in all studies. For infection assays 12 aliquots of 100ul of this cell suspension were incubated with stationary phase (90% metacyclic) *L.major* PM in a 1:5 ratio. The optimal incubation period for maximal infection rate was determined from a time course, sampling cell co-cultures at 2 hourly intervals up to 48 hours.

2.5.2 Fluorescence studies

Double labelling of DC which had taken up PM was visualised with fluorescence microscopy and analysed with flow cytometry on a FACScan (Becton-Dickinson, San Jose, CA) using CELLQUEST software (Becton-Dickinson). The PM and DC were labelled separately as described below.

2.5.2.1 PM labelling

(i) PKH2

Initially a green fluorescent cell linker molecule, PKH2, which is incorporated into the membrane lipid bilayers of cells, labelled PM efficiently. However this labelling was not stable. Repeated vigorous washing of labelled PM did not

prevent PKH2 being subsequently shed into the media, presumably due to rapid PM cell membrane turnover. This was evidenced by incubating PKH2-labelled PM with unlabelled PM at 37^0C. Within 60 minutes dye was taken up by unlabelled PM (data not shown).

(ii) Intracellular fluorescent probe

A relatively new series of fluorescent probes that are retained in living cells through several generations and not transferred among adjacent cells in a population were adapted for this research by labelling live PM to track their course and ultimate fate in the cell cultures. The PM were labelled with either a red (chloromethyl-tetramethyl rhodamine: CMTMR, Biosciences, Cambridge, UK) or green (chloromethyl fluorescein diacetate: CMFDA, Biosciences, Cambridge, UK) intracellular dye. These molecular dyes cross the cell membrane and combine with intracellular thiols to form cell-impermeant thioether adducts which fluoresce following cleavage by intracellular esterases.

The concentration of dye for optimal in vitro labelling with maximal viability was initially ascertained by dose titration curves with flow cytometry and quantification of motile fluorescing PM with fluorescence microscopy. First, serial dilutions of CMTMR or CMFDA were made by diluting 1ul of 10mM dye stock in varying volumes of Dutch modification of RPMI 1640 medium supplemented with 10% vol/vol FCS, 100 IU/ml penicillin and 100μg/ml L-glutamine. Approximately 10^6 stationary phase PM were washed in serum free media then incubated with serial dilutions of dye for 20 minutes. They were then

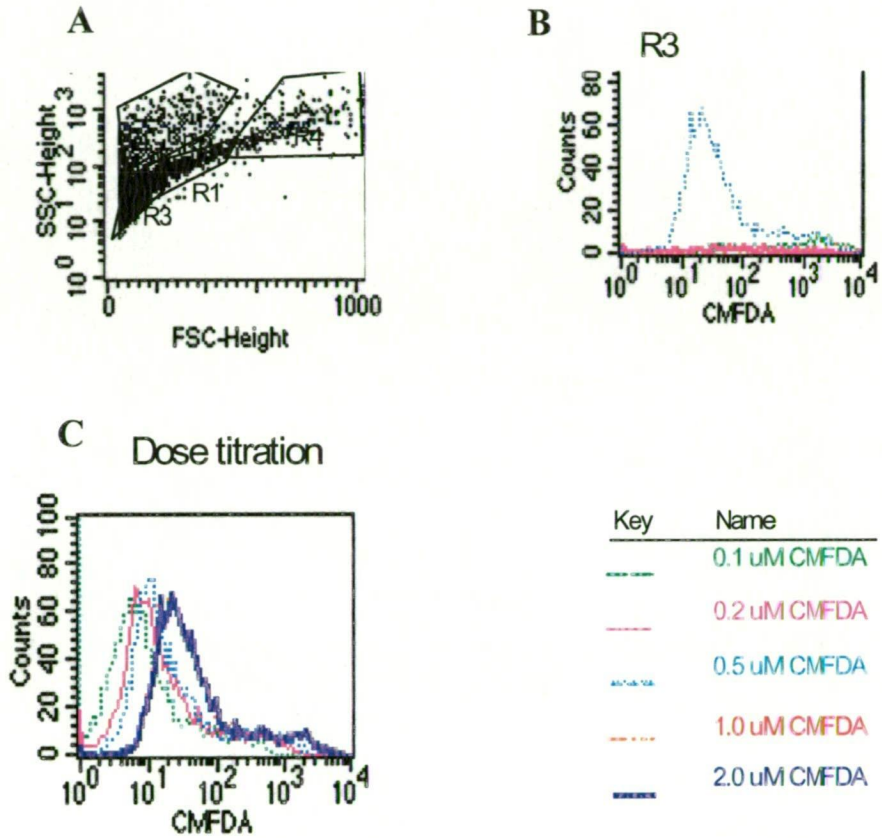


Fig 2.9 Establishment of optimal CMFDA labelling of PM. A. Dot plot of PM culture where Region 3 (R3) comprises the majority of PM, being cells of small size and little granularity. B. Histogram confirming the highest level of CMFDA labelling in R3. C. Dose titration curves established that 2.0μM CMFDA labelled live stationary phase PM most efficiently without loss of PM viability.

washed twice and resuspended in the supplemented RPMI Dutch media and incubated at room temperature for a further 30 minutes. Following two subsequent washes they were ready for use in experiments involving FACS and fluorescence microscopy. These experiments established that a concentration of 2.0uM CMFDA or CMTMR produced a high level of labelling without loss of parasite viability (Fig 2.9).

To ensure no leakage of dye occurred during the period of the cell cultures, labelled PM and unlabelled PM were incubated together at 37^0C, such that any leakage of dye from CMFDA-labelled PM would be detectable by flow cytometry as merging of both unlabelled and labelled curves. There was no dye extrusion into the media from labelled PM for at least 48 hours (Fig 2.10).

Fluorescent labelled PM were incubated with DC and macrophages in a ratio of 5:1 for variable periods of time from two to 48hours. The cells were then labelled with monoclonal antibodies for further identification and prepared for fluorescence microscopy and flow cytometry as described below.

2.5.2.2 DC labelling

Following incubation with PM, the DC and macrophages were identified by labelling with a specific DC monoclonal antibody, NLDC-145 or the specific macrophage marker, F4/80 respectively and indirect conjugation with FITC. The co-cultured DC and PM were washed in cold FACS buffer then divided into

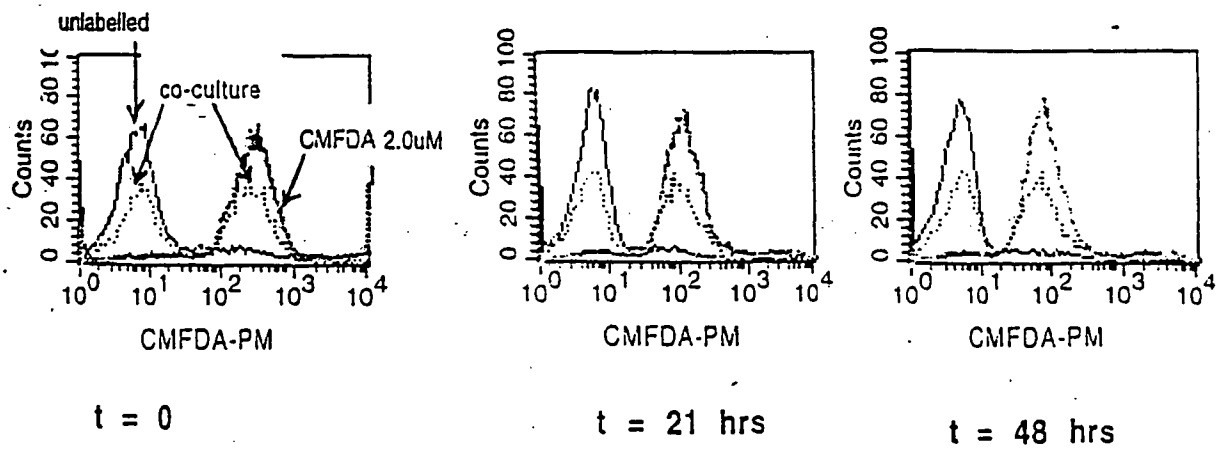


Fig. 2.10 *L.major* PM labelled with 2.0uM CMFDA were incubated with unlabelled PM in a co-culture (dotted line) for 48 hours and level of fluorescent labelling documented by the histograms. Persistent complete separation of the curves and maintenance of a high level of fluorescence when compared to CMFDA labelled PM cultured alone indicate total retention of dye for at least 48 hours.

100µl volumes at a concentration of $5x10^6$ cells/ml. Rabbit serum (20%) was added for 20 minutes to reduce non-specific binding of the subsequent mAb.

NLDC-145 5µl and F4/80 50µl were added separately to DC and macrophage cell suspensions for 20 minutes. Rat IGg2a and rat IGg2b were the isotypes used for NLDC-145 and F4/80 respectively. After 2 washes in the cold centrifuge, 2µl of the second layer anti-rat Ig-FITC (DAKO) was added for 20 minutes. Following two further washes, the cells were then fixed to microscope slides with methanol after cytocentrifugation for five minutes at 600g.

2.5.2.3 Fluorescence microscopy

Glycerol (10%) was added to the slides prepared above and a coverslip fixed so that the cells could be visualised under oil immersion with 100x objective on the inverted fluorescence microscope fitted with filter blocks for dual fluorescence (Zeiss Axioscop). Those cells labelling green with NLDC-FITC containing intracellular red (CMTMR) fluorescing parasites were recorded as *Leishmania*-positive DC (Fig 2.11). Similarly, those cells labelling green with F4/80-FITC containing intracellular red (CMTMR) fluorescing PM were recorded as *Leishmania*-positive macrophages. The rate of *Leishmania* uptake was indicated by the number of parasites observed per cell.

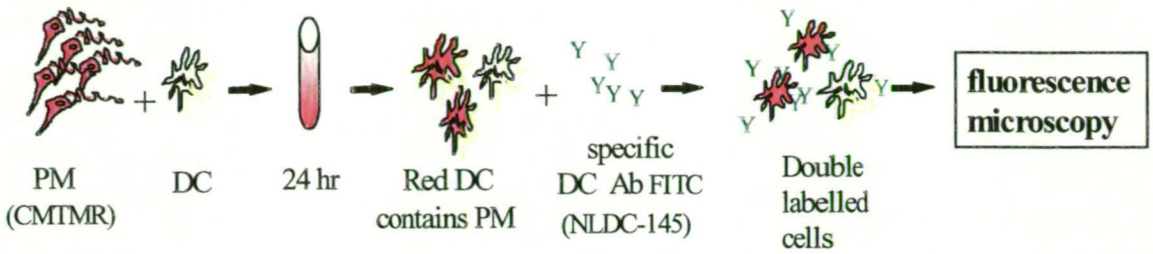


Fig 2.11 Fluorescent labelled PM (CMTMR) are cultured with DC which were identified subsequently with a fluorescing specific DC marker (NLDC-145 FITC). PM internalised by DC can then be identified as double fluorescent labelled cells with inverted fluorescence microscopy.

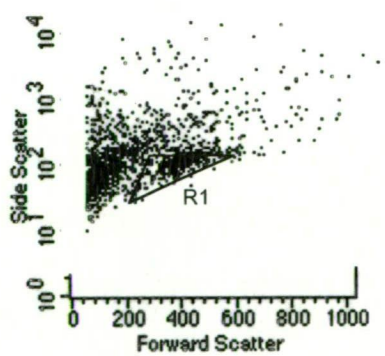


Fig 2.12. Dot-plot of murine splenic LDC after 24 hours culture. The DC population are selected by their larger size and relative lack of granularity and comprise region 1 (R1).

2.5.2.4 Fluorescent Activated Cell Scan (FACS)

Flow cytometry was performed on a FACS (Becton-Dickinson, San Jose, CA) machine and data analysed using CELLQUEST software (Becton-Dickinson).

Dead cells were gated out on the basis of light scatter. DC were analysed by flow cytometry using a gate set on large agranular cells (Fig 2.12). CMTMR was used initially for fluorescence microscopy but the emission characteristics of the CMTMR probe precluded its use with flow cytometry. CMFDA alone was used for PM labelling for FACS analysis. The PM were labelled as described above.

2.5.3 Electron microscopy

Processing of samples for electron microscopy was performed by Mr Nicholas English in the APRG laboratory. To investigate DC internalisation of PM, DC and PM were incubated together in a ratio of 1 DC : 5 PM for varying periods of time from 4 to 48 hours. The optimal time period for analysis was identified at 24 hours.

The DC:PM co-cultures at 24 hours were pelleted and fixed in 3% gluteraldehyde (Agar Scientific, Cambridge, UK), pH 7.4 at room temperature for two hours. Cells were washed in phosphate buffer, placed in 1.5% low-melting point agarose and post-fixed in 1% osmium tetraoxide for 60 minutes at room temperature. The cells were washed overnight in distilled water. Block staining was then performed with 2% uranyl acetate in water for 4 hours in the dark, washed again and

dehydrated with graded acetone. The samples were then infiltrated with Spurr resin overnight, then embedded and cured at 65^0C for 18 hours. The sections were stained with lead citrate and examined under a Jeol 100 CX transmission electron microscope (Tokyo, Japan) at 80 kV.

DC and contaminating macrophages were identified on the basis of their characteristic morphology. DC were identified by their characteristic elongated indented nucleus, paucity of intracellular organelles and long dendritic veils. Macrophages lacked dendritic processes and had a heterogeneous cytoplasm with a variety of organelles and significant numbers of lipid vacuoles. DC containing parasites were quantified and categorised according to the number of parasites per cell, maturity of DC, and viability of the DC and parasite. Over 100 cells were counted and the experiment repeated 3 times. DC from both BALB/c and C57\BL10 mice were used to compare the rate of uptake and persistence of the parasite. Macrophages provided the positive control for parasite uptake and intracellular viability.

In some preparations DC were further identified by immunogold labelling. Following 24 hour incubation with PM, the cells were washed in cold FACS buffer and divided into 100µl aliquots at a concentration of $5x10^6$ cells/ml and labelled with either NLDC-145 20µl or the isotype IGg2a 20µl. After 30 minutes on ice, the cells were washed twice and the second layer goat anti-rat immunogold 20nm applied at a dilution of 1:35. These were washed twice again after 30 minutes. The labelled cells were then pelleted, fixed with 3% gluteraldehyde and underwent the same processing procedure and categorising as above.

2.6 Phenotypic analysis of DC

To analyse the impact of *L.major* PM on DC, a battery of monoclonal antibodies (Table 2.2) were used to assess the DC surface molecule expression including MHC Class I and II, costimulatory molecules and adhesion molecules at various time points in 48 hour co-culture time course experiments. DC were resuspended in cold FACS buffer on ice. The cells were labelled for 15 minutes with pre-determined optimal concentrations of fluorochrome-conjugated mAbs to detect specific cell surface antigens.

All antibodies were purchased from PharMingen, San Diego, CA except for NLDC-145 (George Kraal, Free University, Amsterdam) and CD44 (Serotec Ltd., Oxford, GB). Isotype-matched control labelling was included in all experiments.

As surface molecule expression on DCs alters with DC maturation, DC were exposed to *L.major* PM at various stages of maturity after removal from 'overnight incubation' at time periods from 6 to 24 hours.

To look more specifically at *L.major* PM infected DC, CMFDA labelled PM were co-cultured with DCs and analysis performed with flow cytometry to measure mean fluorescence intensity of the above fluorochrome labelled mAbs on CMFDA+ve DC.

Table 2.2 Phenotypic markers used to identify specific cell types

Surface marker	Clone	Isotype
$I\text{-}A^k$ (C3H MHC class II)	11-5.2	Rat IgG2b
$I\text{-}A^d$ (Balb/c MHC class II)	AMS-32.1	Rat IgG2b
$I\text{-}A^{b,d}$ (C57/B10 MHC class II)	25-9-17	Rat IgG2b
$H\text{-}2D^d$ (Balb/C MHC class I)	34-2-12	Rat IgG2a
$H\text{-}2D^k$ (C3H MHC class I)	15-5-5	Rat IgG2a
CD11a (LFA-1)	2D7	Rat IgG2b
CD11b (Mac-1)	M1/70	Rat IgG2b
CD54 (ICAM-1)	3E2	Hamster Ig
CD11c (integrin αx chain)	HL3	Hamster Ig
CD40	3/23	Rat IgG2a
CD44 (Pgp-1, Ly-24, H-CAM)	IM7	
CD80 (B7/BB1)	1G10	IgG2a
CD86 (B7-2)	GL1	IgG2a
CD40 (ligand gp39)	MR1	IgG2a
DEC205 (NLDC-145)		IgG2a
CD3 OKT3		Hamster IgG
CD19		Rat IgG2a

2.7 Primary lymphocyte proliferation assays

The function of DC after exposure to *L.major* was determined by their capacity to stimulate proliferation of syngeneic lymphocytes. Hanging drop cultures were used to measure antigen specific T cell responses as previously described (Knight SC 1987).

In brief, enriched DC or macrophages were incubated with washed metacyclic *L.major* PM (cell to parasite ratio of 1:5) for 4 hours. Control DC and macrophages were incubated with medium alone. After washing, graded numbers (1000, 2000 and 4000/well) of these stimulator cells were cultured with varying numbers of lymph node cells (25 000, 50 000 and 100 000 LNC/well) in triplicate 20 µl hanging drops in inverted Terasaki plates. The plates were inverted over saline and incubated for 3 days in a humidified atmosphere of 5% CO_2 in air at 37^0C. They were then pulsed for 4 hours with 1µl (equivalent to 1µg/ml thymidine at a specific activity of 2 Ci/mmol) [^{3}H] thymidine (Amersham International, UK) and harvested by blotting onto filter paper discs. The acid-insoluble material was counted using a liquid scintillation counter (Fig 2.13). This technique using low specific-activity thymidine in flooding conditions for a short pulse time results in low counts but these reflect the level of DNA synthesis without the complication of limiting availability of thymidine or excessive radiation damage (Knight SC 1987). Radioisotope incorporation was determined by liquid scintillation counting.

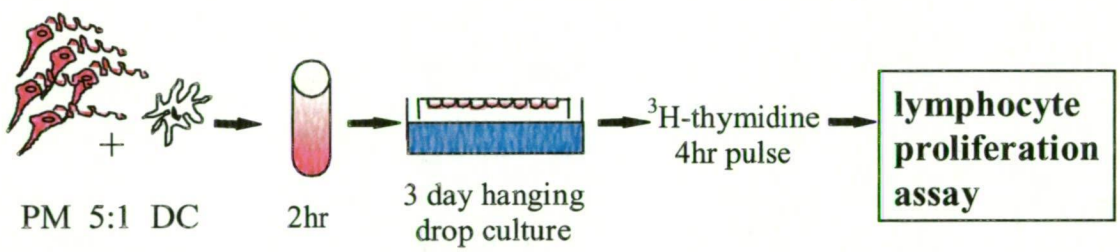


Fig 2.13 DC pulsed with PM for two hours were used in 3 day hanging drop cultures with syngeneic LNCs incorporating ^{3}H-thymidine after which they were harvested and lymphocyte proliferation measured by counts per minute (cpm) on a scintillation counter.

In later experiments, particularly where supernatant cytokine analysis was performed, the lymphocytes were enriched for T cells by incubating LNC suspensions over sterilised nylon wool columns at 37^0C for 45 minutes to remove B cells as described above.

Supernatant was collected from stationary phase PM and passed through a 0.2uM filter. DC were pulsed with *L.major* culture supernatant (20% vol/vol) for 2 hours, washed twice then co-cultured with syngeneic lymphocytes using the hanging drop technique detailed above. Similarly, lymphocyte proliferation was measured by incorporation of ^{3}[H] thymidine.

2.8 Cytokine Analysis

2.8.1 IL-12 production by DC

A two-site sandwich enzyme-linked immmunosorbent assay (ELISA) (BioSource International, Camarillo, USA) technique was used to detect the production of IL-12p40 by DC stimulated with *L.major* PM. In a sandwich ELISA, the first antibody, which recognises the first epitope, binds to the bottom of a plastic well. The interaction of the cytokine with this antibody is demonstrated with a second antibody that binds on a different epitope on the cytokine. An enzymatic colorimetric assay was used to measure the reaction. The optical density was read with an ELISA-reader (Eurogenetics) and concentrations were calculated by means of a standard curve.

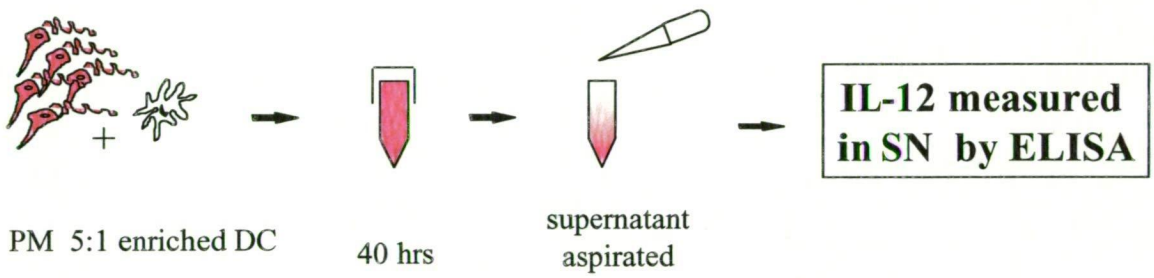


Fig 2.14 NLDC-145 or CD11c selected DC were co-cultured for 40 hours with PM. The supernatant from the co-culture was analysed for IL-12p40 production using an ELISA technique.

It was imperative to ensure minimal macrophage and other IL-12-producing cell contamination for these sensitive assays. NLDC-145 or CD11c selected DC from both strains of mice (approximately 5 x 10^6/ml) were incubated with PM at a ratio of 1:5 in 200μl complete medium for approximately 40 hours. Cultures were centrifuged through 0.22μm filters (Spin-X tubes, Costar, Cambridge, USA) for 5 minutes and the supernatant assayed by ELISA (Fig 2.14). The lower limit of sensitivity for the IL-12p40 assay is defined by the manufacturers as the lowestconcentration of standard which shows absorbance greater than the mean absorbance of 0 pg/ml sample +2 standard deviations. It was calculated to be 7.8 pg/ml.

2.8.2 Supernatants from lymphocyte proliferation assays

Supernatants from the cell cultures of the hanging drops were harvested on day 3 of culture. In parallel with the lymphocyte proliferation assays described above, 60 well Terasaki plates were used to collect supernatant from 30 x 20ul wells of the following cell culture per 20ul well: 10^5 syngeneic LNCs alone, 4000 DC co-cultured with 10^5 syngeneic LNCs , and 4000 DC pulsed with PM co-cultured with 10^5 syngeneic LNCs. These "cytokine plates" produced samples of cell suspensions for "LNCs alone, LNCs + DC, LNCs + DC + PM and DC + PM". The cell suspension collected from 30 x 20ul wells was centrifuged at 6500 rpm for 5 minutes. The supernatant was aspirated with fine-tipped pipettes and stored separately at -70^{0}C for batch testing with ELISA kits as described below. The cell pellets were resuspended in Tri-reagent and frozen at -70^{0}C for later mRNA analysis for IL-4, IFN–γ and IL-12.

IFN–γ and IL-4 were measured with the InterTest™ Mouse IFN–γ and IL-4 ELISA kits (Genzyme diagnostics, Cambridge, USA) according to the manufacturers instructions. Briefly, these were solid phase sandwich ELISA kits with 96 well microtitre strips coated with the cytokine specific antibody. Samples, including standards of known cytokine content, control specimens and unknown supernatants were pipetted into these wells in 100µl volumes in duplicate, followed by the addition of a biotinylated second antibody. During the first one hour incubation, cytokine antigen binds simultaneously to the immobilised (capture) antibody on one site and to the solution phase biotinylated antibody on a second site. After removal of excess second antibody, streptavidin-peroxidase was added and binds to biotinylated antibody to complete the four-member sandwich. After a second one hour incubation followed by washing to remove all of the unbound enzyme, a substrate solution was added, initiating a peroxidase catalysed colour change that was subsequently stopped by acidification. The absorbance measured on an ELISA reader at 450 nm was proportional to the concentration of cytokine present in the standards or supernatant. A standard curve was constructed by plotting the concentrations of the cytokine standards against the respective absorbance. The curve was non-linear and four-parameter regression analysis provided the best fit to the data, as per manufacturers instructions. The concentration in the supernatants was then determined using the standard curve after background absorbance of control medium had been subtracted. According to the manufacturer, the detection limit for both assays was determined to be 5 pg/ml after statistical analysis of ELISA results. The mean absorbance obtained with 5 pg/ml mIL-4 was greater than two standard deviations

above the mean baseline absorbance obtained form replicate zero control wells (wells containing all assay components except mouse IL-4 or IFN-γ).

IL-12 was also measured in the supernatants using an ELISA kit to detect the production of IL-12p40 (BioSource International, Camarillo, USA) as described above.

Using these assays on supernatants from the lymphocyte assays enabled analysis of the effect of PM-pulsed DC on T cell cytokine production, particularly IL-12, IL-4 and IFN-γ, in both susceptible and resistant strains of mice.

2.9 Cytokine blocking assays (anti-IL-12)

To determine if T cell proliferation was driven by DC derived IL-12, further analyses were performed using anti-IL-12 monoclonal antibody (clone C17.15; Genzyme diagnostics, Cambridge, USA) to neutralise IL-12 in the lymphocyte proliferation assays. This mouse antibody recognises the p40 subunit of mouse IL-12 either as a monomer, a homodimer or as a part of the p70 heterodimer. A concentration of approximately 1.0 μg/ml was recommended by the manufacturers to adequately neutralise the proliferative effect of 20pg/ml of recombinant mouse IL-12.

Lymphocyte proliferation assays were set up with *L.major* PM-pulsed-DC as described above. An additional identical set of Terasaki plates were set up in parallel to the standard plates and each well was pulsed with 2 μl of anti-IL-12

antibody. The effect on proliferation was measured with the thymidine incorporation method as outlined and compared to control samples.

2.10 Non-peptide antigen presentation by DC

2.10.1 *L. major* supernatant fractionation

Using the in vitro model above, it was established that DC could stimulate primary T cell proliferation. This finding prompted the question of whether DC pulsed with non-peptide antigen from the supernatant were capable of producing a similar response.

To obtain protein free supernatant, stationary phase supernatant was treated with pronase. Parasite-free culture supernatant was obtained, as described above, from the stationary phase cultures by centrifugation through a 0.22μm filter at 600g for 5min. The supernatant was desalted by dialysis overnight in distilled water using a slide-A-lyzer kit with a 10 000 MW threshold. To digest the protein, approximately 10 μl of pronase (Sigma) was used for 1 mg of protein as recommended. The salt free supernatant dialysate had an estimated protein content of 20mg/ml and 1 ml was therefore incubated with 200μl of pronase for 48 hours at 37^0C. Pronase self digests such that any residual pronase would have been eliminated by 48 hours. [4]

[4] There are some filamentous proteoglycans which are predominantly carbohydrate but do have a protein backbone of alanine, proline and serine, which are notoriously resistant to proteases (reviewed in Ilg 2000). Therefore to exclude residual protein in the pronase treated supernatants they would need to be analysed by HPLC.

2.10.2 Fractionated protein in lymphocyte proliferation assays

To establish whether this protein depleted supernatant was immunogenic, DC were pulsed with 10ml aliquots, and used in 3 day syngeneic lymphocyte proliferation assays, quantifying the level of thymidine incorporation on a scintillation counter as outlined above. The controls used were DC alone and DC pulsed with unfractionated stationary phase supernatant.

2.11 NLDC-145 Blocking Experiments

To investigate whether DEC-205 was a major pathway for the uptake of *L.major* PM by DC, the effect of blocking DEC-205 receptors with NLDC-145 antibody was studied. NLDC-145 antibody treated DC subsequently exposed to *L.major* PM were analysed by light microscopy to quantify internalised PM and determine if there was a reduction in the number of PM internalised compared to controls. Additionally NLDC-145 treated DC pulsed with *L.major* PM were used in lymphocyte proliferation assays to determine if there was any reduction in immunostimulatory capacity of thus treated DC.

Specifically, BALB/c and C57/BL10 DC at a concentration of approximately 5 x 10^5 in 100 µl volumes were pulsed with a range of 20 - 30µl of NLDC-145 antibody for 1 hour prior to the addition of stationary phase *L.major* PM in the standard ratio of 1 DC: 5 PM. The DC:PM co-cultures were then incubated together for 24 hours with sampling at 4 hourly intervals for light microscopic quantification. The samples were compared to control DC pulsed with 20 µl of

isotype control antibody IgG2a antibody. NLDC-145 antibody treated DC and isotype control antibody treated DC were pulsed with stationary phase *L.major* PM for 2 hours in the same ratio for 3 day lymphocyte proliferation assays using the method outlined above.

CHAPTER 3

RESULTS

RESULTS

3.1 DC internalise *L.major* PM

3.1.1 Light microscopy

DC were cultured for 48 hours with stationary phase *L.major* PM in a ratio of approximately 1 DC:5 PM. The Geimsa stained cytospin preparations of the co-culture sampled at approximately 4 hour intervals were quantitatively analysed for Dc uptake of PM. Approximately 100 cells were analysed per sample and the data represents the average of 5 experiments. DC were identified by their morphological appearance. They had a relatively homogeneous cytoplasm with elongated, indented nuclei and several long dendritic processes extending from the cell (Fig 3.1). Promastigotes were counted as internalised in the cell if whole or part thereof was identified (Fig 3.1). Uptake of PM was observed as early as 2 hours after incubation. Maximal uptake occurred at approximately 24 hours as shown in the time course experiments (Fig 3.2).

The percentage of DC observed to have internalised promastigotes as measured by light microscopy of Geimsa stained cytospin preparations of DC:PM co-cultures peaked at approximately 25%. Parasites numbered from one to three per cell. This contrasted strikingly with the appearance of the contaminating macrophages which were larger, with 10-20 AM in multiple parasitophorous vacuoles and which generally had more vacuolated, coarse, heterogenous cytoplasm. Furthermore even

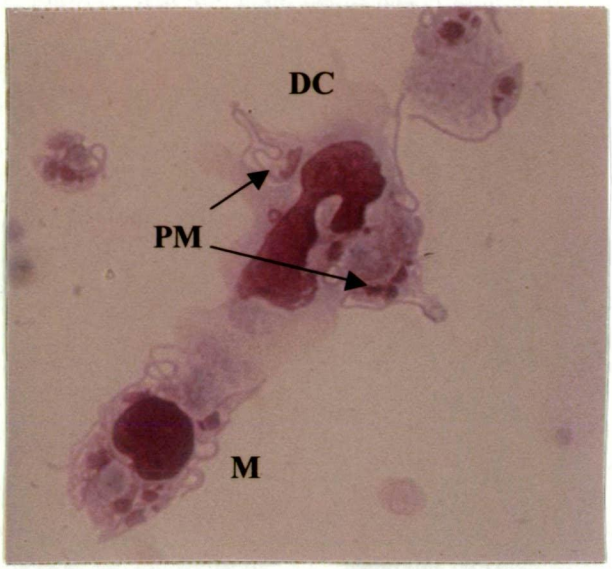


Fig 3.1 Geimsa stained cytospin preparation of DC:PM co-culture at 24 hours demonstrating a DC with characteristic features of elongated horseshoe nucleus, dendritic processes and 3 PMs in the process of being internalised. The adjacent cell is a macrophage (M) with a rounded nucleus containing >5 PMs.

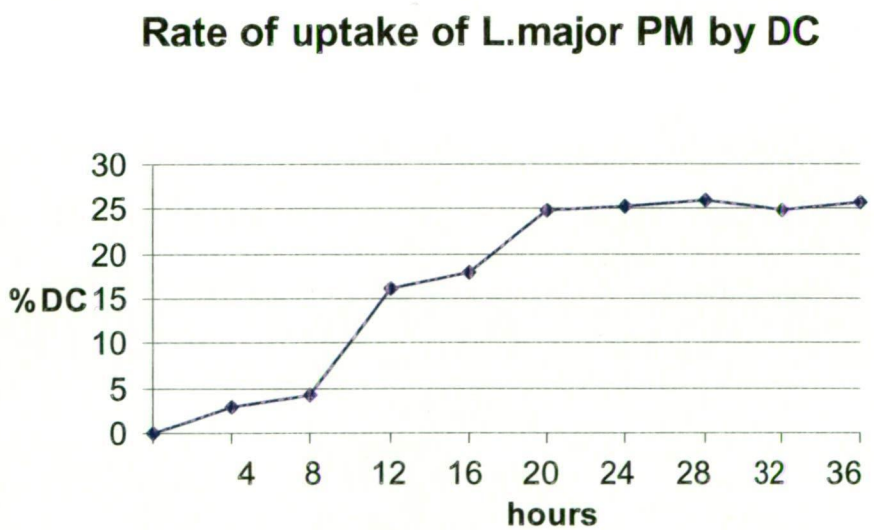


Fig 3.2 Percentage of DC with internalised PM measured at 4 hourly intervals using Geimsa stained cytospin preparations of DC:PM co-cultures peaked at approximately 25% at 24 hours.

with sampling co-cultures for 5 days, AM were never seen within DC. No difference was observed between strains of mice.

Interestingly, in many of the slide preparations close apposition of macrophages to DC, the latter often wrapped around the macrophage, was frequently observed (Fig 3.1). The research schedule did not allow for investigations to further explore this observation but cross-talk between these two cell types, would appear to be highly likely.

3.1.2 Fluorescence studies

To corroborate this novel finding of *L.major* PM internalisation by DC, particularly with respect to further confirmation of DC identity, fluorescence microscopy and flow cytometry were employed. Fluorescence microscopy was another modality used to visualise parasite internalisation, with additional labelling of cells by fluorescent tagged, specific mAbs to differentiate DC from macrophages. Using flow cytometry large numbers of cells could be more accurately assessed to determine how many cells internalised parasites.

3.1.2.1 Fluorescence microscopy

Co-cultures of DC labelled with NLDC-145-FITC (green) and PM labelled with CMTMR (red) were sampled at 48 hours for cytospin preparation. The cells were visualised with an inverted fluorescence microscope using red and

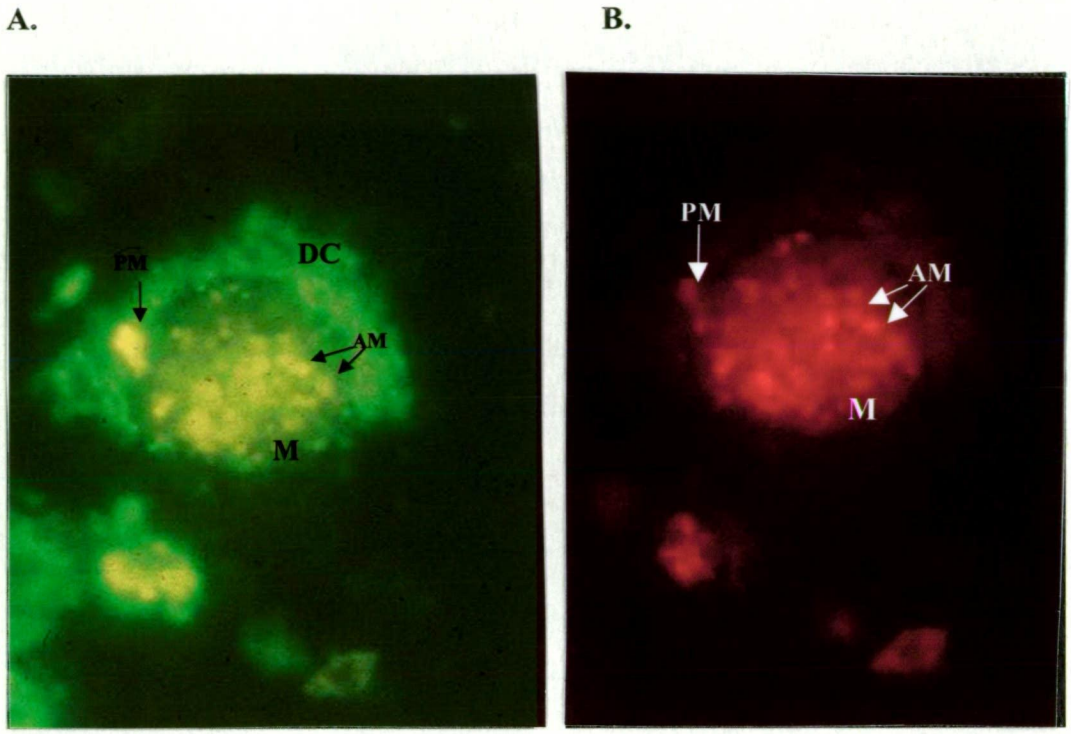


Fig 3.3 Fluorescence microscopy demonstrating a DC encircling a macrophage (M) at the superior pole in this view. **A**. The DC labelled positively with NLDC-145 FITC appearing as diffuse irregularly shaped apple green capping over the macrophage which did not label with NLDC-145. **B.** The promastigote (PM) and amastigotes (AM) fluoresced red with CMTMR when viewed through the green filter. Thus they appeared yellow when the red filter was used (as seen in **A**). A singular PM was visible in the DC cytoplasm. As occurred in the light microscopy studies, no AM were seen within DC. By comparison, the macrophage is packed with >10-20 AM.

green filters (Zeiss Axiosxop). Whole parasites could be seen within DC. The DC labelled positively with NLDC-145 FITC and appeared as diffuse, irregularly shaped, apple green speckled cells either alone or occasionally in close apposition with a macrophage when viewed through the red filter. The PM and AM labelled with CMTMR could be seen fluorescing red when viewed through the green filter. When viewed through the red filter, PM and AM appeared yellow. Macrophages were identified by the large number of intracellular AM and lack of NLDC-145 staining.

Approximately 20% of NLDC-145^{+}cells had evidence of *L.major* PM uptake typically numbering between one and three PM per cell. As has occurred in the light microscopy studies, no AM were seen within DC. By comparison, the contaminating macrophages frequently contained >10 AM (Fig 3.3).

Macrophages did not consistently and reliably label with the macrophage specific monoclonal antibody F4/80. This may be explained by the observation in original work with the antibody, that the state of cell activation modulates the expression of the F4/80 molecule with antigen stimulated macrophages generally expressing low levels (Austyn and Gorden 1981). In this work macrophages were readily identified by the cell morphology and the large number of intracellular AM.

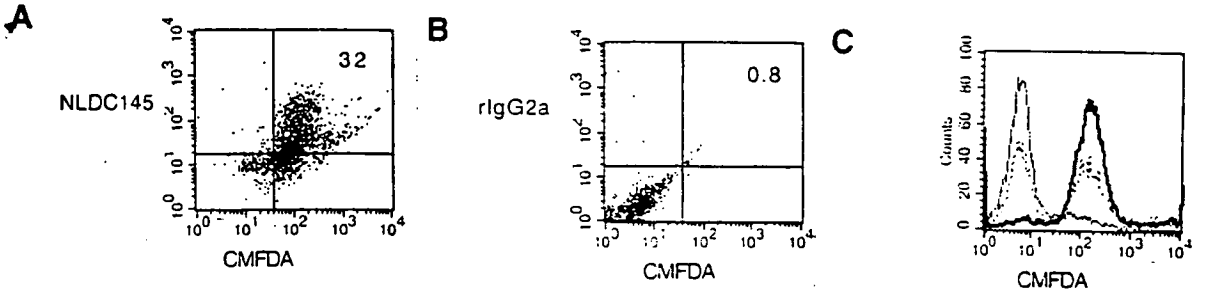


Fig 3.4 Flow cytometric evidence of internalisation of *L.major* PM by DC. Spleen DC were incubated with CMFDA-labelled PM for 20hrs, double labelled with NLDC-145-PE and analysed by flow cytometry using a gate set on large agranular cells. **A.** NLDC-145^{+} DC acquire fluorescent material (FL1) from CMFDA-labelled PM. **B.** No staining of DC was observed with an isotype-matched mAb or following incubation with unlabelled PM. **C.** Co-culture of labelled and unlabelled PM for 20hrs confirmed absence of spontaneous leaching of dye from PM. (_____) unlabelled PM, (_____ bold) CMFDA-labelled PM, (·····) co-culture of labelled and unlabelled PM for 20hrs.

3.1.2.2 Flow cytometry

Using flow cytometry, the proportion of DC containing fluorescent dye could be quantified from DC co-culture with CMFDA-labelled PM for 20 hours at 37°C. Approximately 30 % of the total cell population and 80% of the NLDC-145$^+$ cells were CMFDA positive (Fig 3.4A,B). Therefore, by this method approximately 80% of DC had evidence of PM internalisation.

This was a significantly higher percentage than was observed with light or fluorescence microscopy. To exclude the possibility of leakage of dye from labelled PM and non-specific uptake by DC, co-cultures of CMFDA-labelled PM and unlabelled PM were established in parallel with the cultures of DC and PM. By the end of the culture period there was no evidence of acquisition of dye by the unlabelled population confirming non-leakage of dye from the labelled PM in culture (Fig 3.4C). This finding supports the suggestion that DC acquire fluorescent material as a result of an interaction with the parasite rather than by uptake of dye released into the medium.

The discrepancy between microscopy and flow cytometry findings may be due to rapid processing of PM by DC following internalisation of the parasite, releasing the fluorescent dye intracellularly resulting in a positively CMTMR staining DC without intact PM. This is supported by the absence of AM-containing DC and suggests a more hostile intracellular environment not permissive to parasite survival and replication. There were no differences noted between strains of mice.

3.1.3 Electron microscopy

To clearly delineate the intracellular uptake of PM and characterise the process, specifically whether they were found in PV, and to determine more definitely if any AM forms were present, electron microscopy of DC:PM co-cultures was performed. Enriched BALB/c and C3H spleen DC of 24 hours maturation were incubated for 24 hours with metacyclic *L.major* PM. The co-culture was then centrifuged and the cell pellet was fixed for electron microscopy. A small contaminating fraction of macrophages enabled direct comparison of DC handling of the parasite with macrophage processing under identical conditions. One hundred cells were counted in each sample and the DC and macrophages, identified morphologically and by presence or absence of specific NLDC-145 labelling, were enumerated and expressed as a percentage of the total cell count. The number and stage of parasites found within each cell type was documented. The data is representative of 3 experiments.

Approximately 90% of the viable LDC were identified morphologically as DC. Of the contaminating cells, 8% were macrophages and 2% lymphoid blasts. Electron microscopy confirmed DC uptake of *L.major* PM (Figs3.5, 3.6, 3.7). Approximately 30% of DC internalised PM (Fig 3.5). The average number of parasites seen per DC was 2.2 PM. Some of these were seen within phagosomes. Classic PV were not typically observed in DC. No AM forms of *L.major* were observed in cultures maintained for an additional 24 or 48 hours and examined by light microscopy. Many of these cells with DC morphology were NLDC-145^{+} by immunogold labelling, supporting their identification as DC (Fig 3.7).

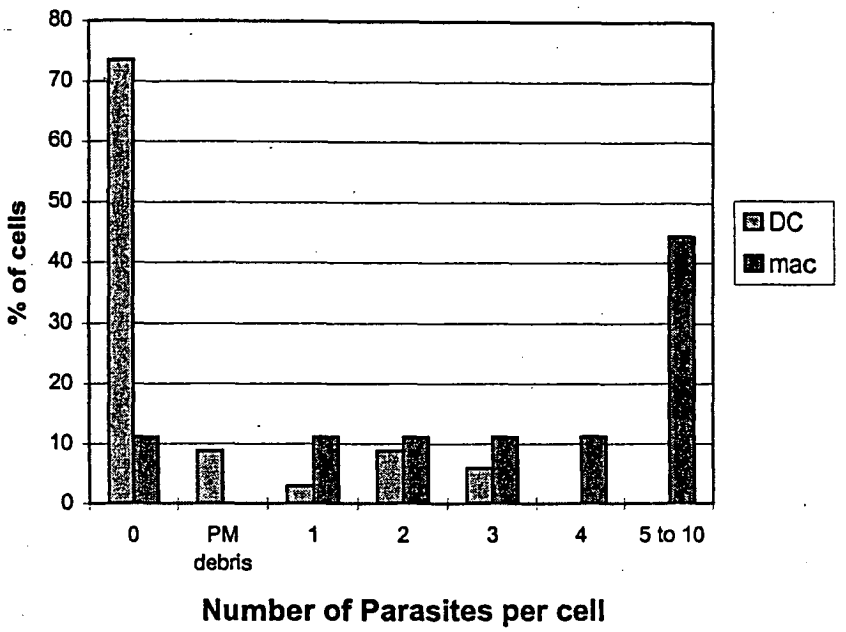


Fig 3.5 Internalisation of *L. major* PM by DC and macrophages (mac). LDC were incubated with PM for 24hrs, fixed and analysed by electron microscopy. The number of internalised parasites was determined for DC and macrophages identified by morphological criteria. Data is representative of 3 experiments.

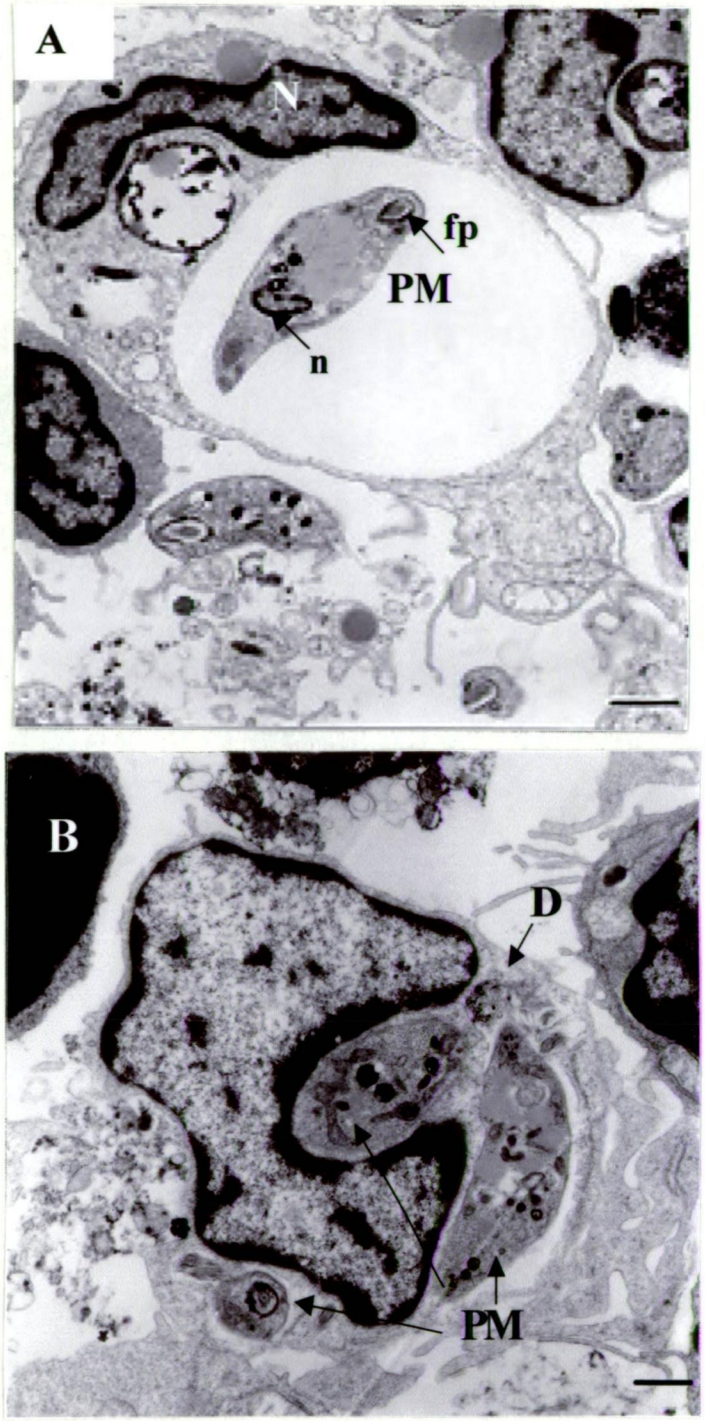


Fig 3.6 Electron micrographs illustrating uptake of *L.major* PM by DC. DC were incubated for 24 hours with metacyclic *L.major* PM. Bar = 1υm. **A**. DC with characteristic indented elongated nucleus (N) and dendritic processes (P) containing a single promastigote (PM) in a well circumscribed phagosome. The PM nucleus (n) and flagellum within flagellar pocket (fp) are demonstrated. Bar=1υm. **B**. DC containing 3 PM and material suggestive of degraded PM (D).

Fig 3.7 Electron micrograph of immunogold staining of PM-bearing DC with LDC-145. DC incubated with *L.major* PM for 24 hours labelled with rat mAb NLDC-145 and EM goat anti-rat IgG: 20nm gold (arrows). Bar =1um.

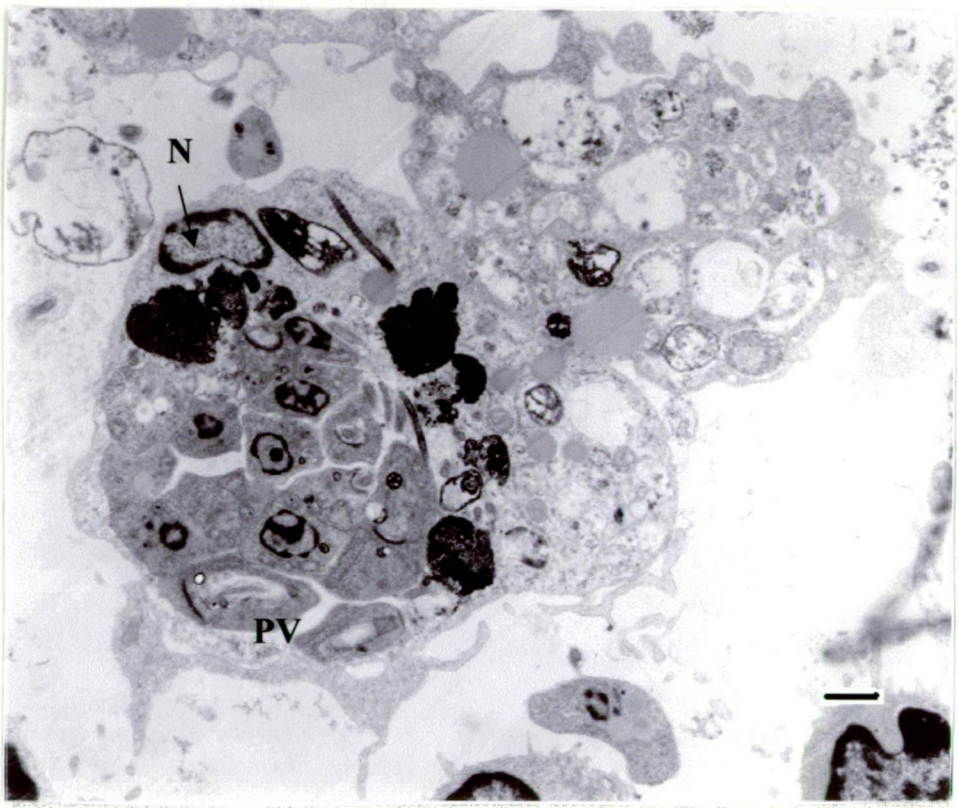


Fig 3.8 Electron micrograph illustrating uptake of *L.major* PM by a contaminating macrophage in a 48 hour co-culture of DC and metacyclic *L.major* PM. Bar = 1um. A macrophage bearing multiple parasites in the process of transforming into AM within a parasitophorous vacuole (PV) seen below the nucleus (N).

In contrast, macrophages contaminating the DC preparation contained a higher parasite load with an average of 8 parasites per cell (Fig 3.5, 3.8). Parasite transformation into AM forms in PV were observed within macrophages (Fig 3.8).

These data suggest that *L.major* PM are internalised by both DC and macrophages and that their fate within these two populations is different. A similar rate and pattern of DC internalisation of *L.major* PM was observed in all strains of mice.

3.1.4 DC surface molecule regulation

DC upregulate certain costimulatory molecules, CD40, CD80, CD86, adhesion molecules ICAM-1 and MHC class I and II molecules with maturation. DC isolated on metrizamide gradients after overnight culture of spleen cells were shown to be a population of mature cells expressing high levels of MHC antigen, co-stimulatory and adhesion molecules. Incubation of these DC with *L.major* PM did not effect the cell surface expression of any of the following molecules as assessed by mAb labelling and flow cytometry: MHC class I and II, CD40, CD80, CD86, CD54, CD44, CD11a, CD11b, CD11c or CD40L (Fig 3.9, 3.10, 3.11).

Five experiments assessing costimulatory, adhesion and MHC antigen expression were performed. To illustrate the impact of *L.major* PM exposure on DC surface molecule expression when DC are exposed at various stages of maturity, the results of selected experiments are demonstrated in Fig 3.9, 3.10, 3.11 below. Results for both susceptible and resistant strains of mice were similar.

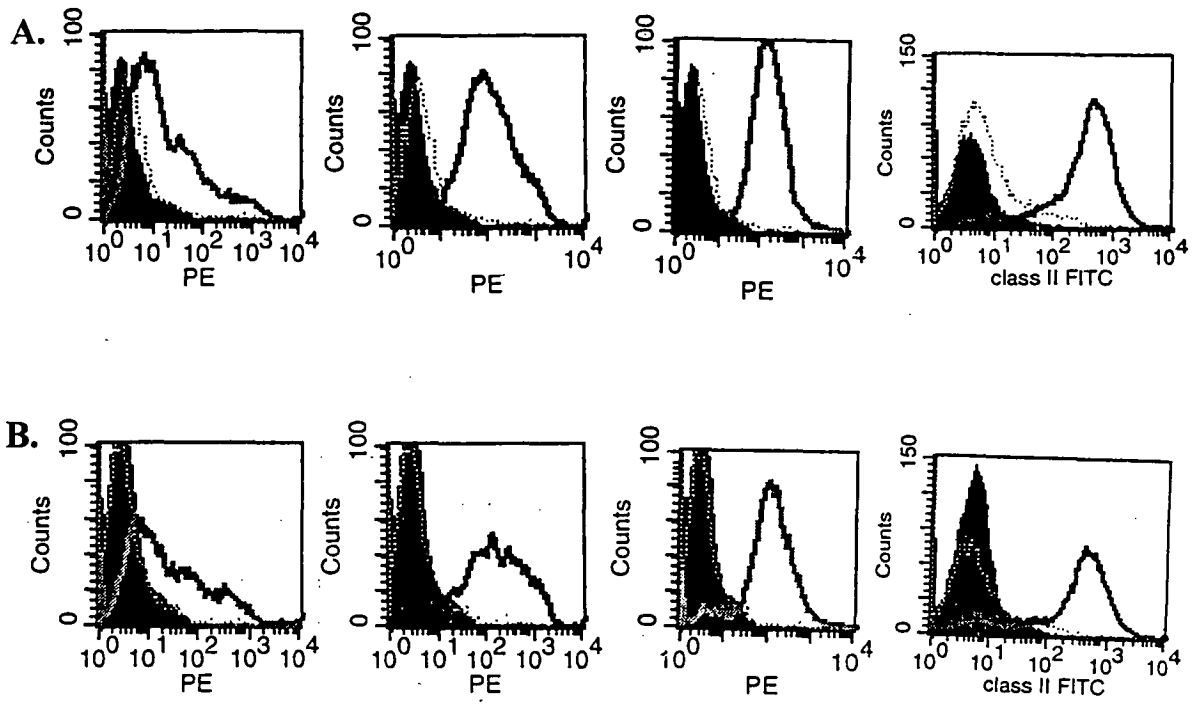


Fig 3.9 Flow cytometric illustration of the effect of *L.major* PM exposure on DC expression of costimulatory molecules CD40, CD80, CD86 and MHC class II when very immature, 2 hour non-adherent spleen LDCs are co-cultured with PM for a further 15 hours. The shaded area represents unlabelled DC, dotted line represents isotype control and the bold line, mAb labelling. **A.** In the control group, after a total of 17 hours culture, there was strongly positive labelling of the DC with CD40, CD80, CD86 and MHC class II. **B.** The addition of PM to the DC did not alter the level of expression of any of these antigens when compared to controls. Similar results were obtained for both resistant and susceptible mice.

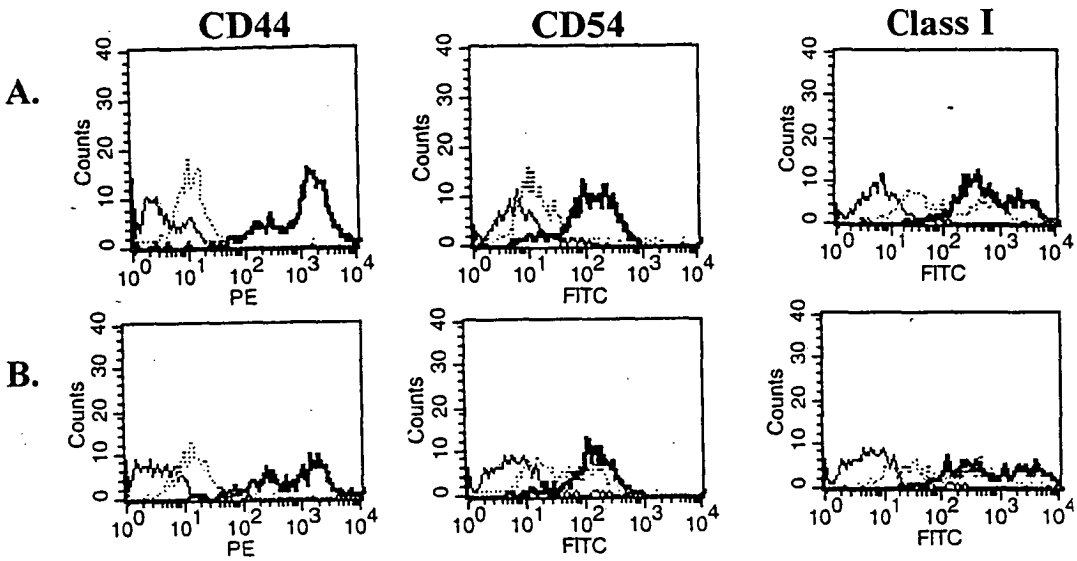


Fig 3.10 Flow cytometric illustration of effect of *L.major* PM exposure on DC expression of CD44, CD54 (ICAM-1) and Class I when DC of 16 hour maturation were co-cultured with *L.major* PM for a further 24 hours. The shaded area represents unlabelled DC, dotted line represents isotype control and the bold line, mAb labelling. **A.** The control DC show a high level of labelling of CD44, CD54 and Class I which persists with prolonged culture. **B.** The DC exposed to *L.major* PM did not have altered level of expression of these antigens. No differences were observed between mouse strains.

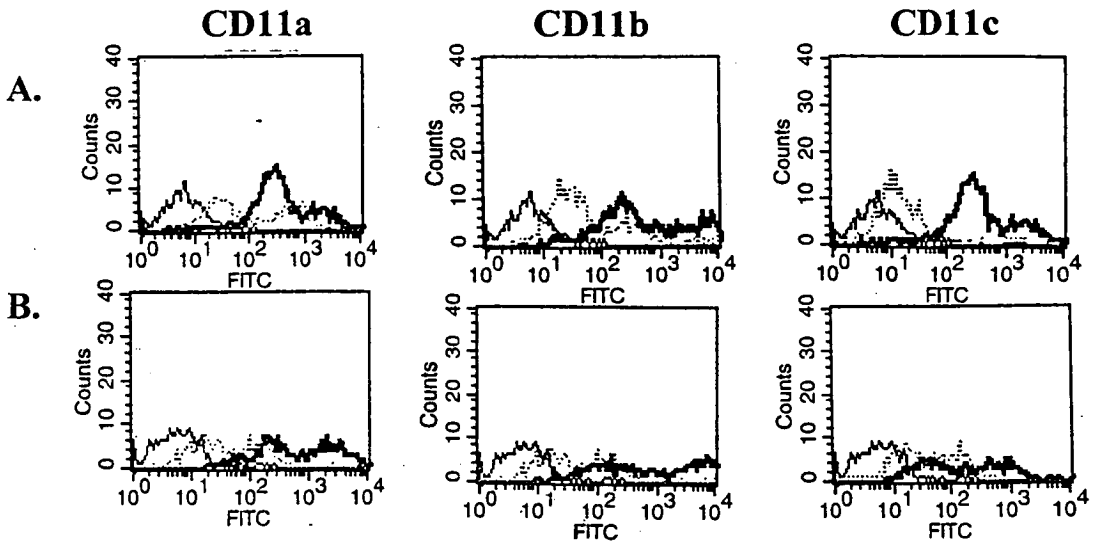


Fig 3.11 Flow cytometric illustration of effect of *L.major* PM exposure on DC expression of CD11a (LFA-1), CD11b (mac-1), and CD11c when DC of 16 hour maturation were co-cultured with *L.major* PM for a further 24 hours. The shaded area represents unlabelled DC, dotted line represents isotype control and the bold line, mAb labelling. **A.** In the control DC there is a high level of co-stimulatory molecule labelling which persists with prolonged culture. **B.** The DC exposed to *L.major* PM did not have altered level of expression of these antigens. No differences were observed between mouse strains.

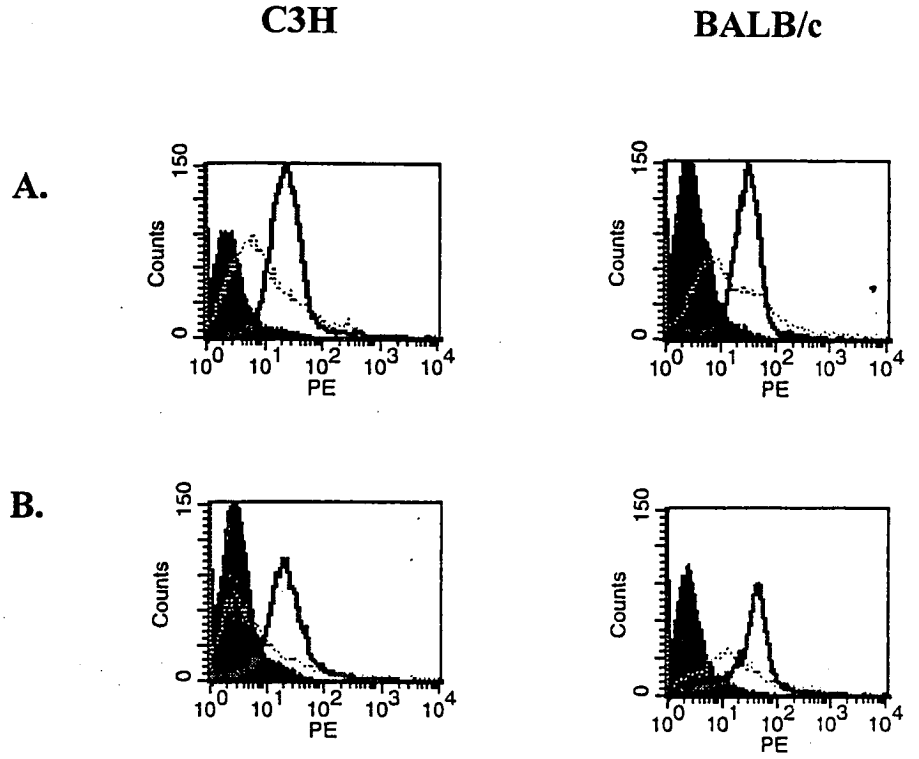


Fig 3.12 Flow cytometric illustration of effect of *L.major* PM exposure on DC expression of NLDC-145 when very immature, 2 hour non-adherent spleen LDCs are co-cultured with PM for a further 15 hours. **A.** After a total of 17 hours maturation, there was positive labelling of the control DC with NLDC-145. **B.** DC exposure to PM did not alter the level of expression of NLDC-145. Similar results were obtained for both resistant (C3H) and susceptible (BALB/c) mice as shown here.

Not all DC internalised PM as demonstrated above. Further analysis of the level of expression of costimulatory, adhesion and MHC antigens by CMFDA+ve DC was performed. There was effectively no difference in the level of surface molecule expression by *L.major* PM-infected DC compared to the whole DC population in the *L.major*-exposed sample (data not shown). DC were able to upregulate their expression of co-stimulatory, adhesion and MHC molecules unaltered by the presence of *L.major* PM.

Due to the potential involvement of DEC205 with DC uptake of PM, the impact of PM exposure on the level of DC expression of NLDC-145 was examined. As was observed with MHC antigen and costimulatory molecules, NLDC-145 expression by DC was not altered by exposure to metacyclic PM (Fig 3.12).

3.2 DC produce IL-12p40 upon stimulation with *L.major* PM

Initial experiments used highly purified NLDC-145 selected DC to assess whether exposure to metacyclic *L.major* PM stimulated DC to produce IL-12. NLDC-145$^+$ DC produced a low level of background IL-12 following a further 40 hour culture period. Exposure to metacyclic *L.major* PM significantly upregulated IL-12 production in both resistant and susceptible murine strains (Fig 3.13).

These experiments were repeated with a different method of selection, using a mAb to CD11c, to ensure that the NLDC-145$^+$ DC were not the only DC subtype capable of producing IL-12 in response to *L.major* PM exposure. In addition, macrophages were cultured simultaneously under identical conditions to determine if macrophages

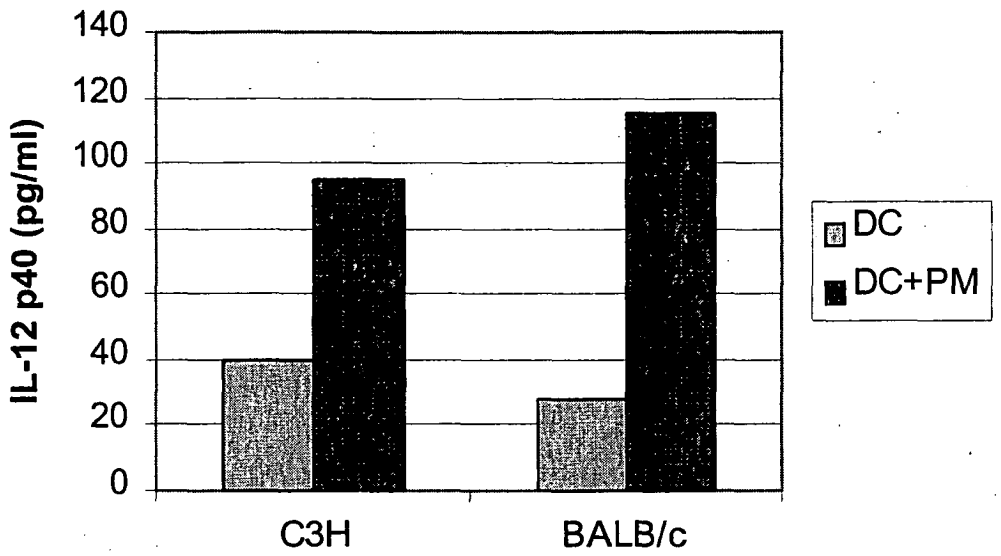


Fig 3.13. *L.major* PM upregulated the production IL-12p40 by NLDC-145 selected DC from BALB/c and C3H mice.

infected by *L.major* PM were contributing to the IL-12p40 being detected in the supernatant (Fig 3.14).

Highly purified $CD11c^+$ DC were stimulated to upregulate the production of IL-12p40 following culture with PM for 48-72hrs (Fig 3.14A). Neither $CD11c^-$ LDC which were depleted of DC (<2% $CD11c^+$), nor adherent macrophages produced IL-12p40 under similar conditions (Fig 3.14A). PM stimulated production of IL-12 by both BALB/c and C3H DC (Fig 3.14B). Low levels of IL-12p40 were also consistently detected in cultures of unstimulated DC and in most experiments these levels were greater in cultures of BALB/c DC than in cultures of C3H DC (Fig 3.13, 3.14).

3.3 Primary Immune Responses

3.3.1 DC stimulate primary T cell responses to *L.major* PM.

DC pulsed with *L.major* PM or with cell-free *L.major* culture filtrates were able to stimulate primary T cell responses (Fig 3.15). The data shown is representative of more than 10 experiments. Primary responses were not consistently higher in one mouse strain than the other.

Despite taking up large numbers of PM, adherent macrophages failed to stimulate naive T cells confirming that this APC population is unlikely to play a role in stimulating primary immune responses (Fig 3.16). The viability of the macrophages

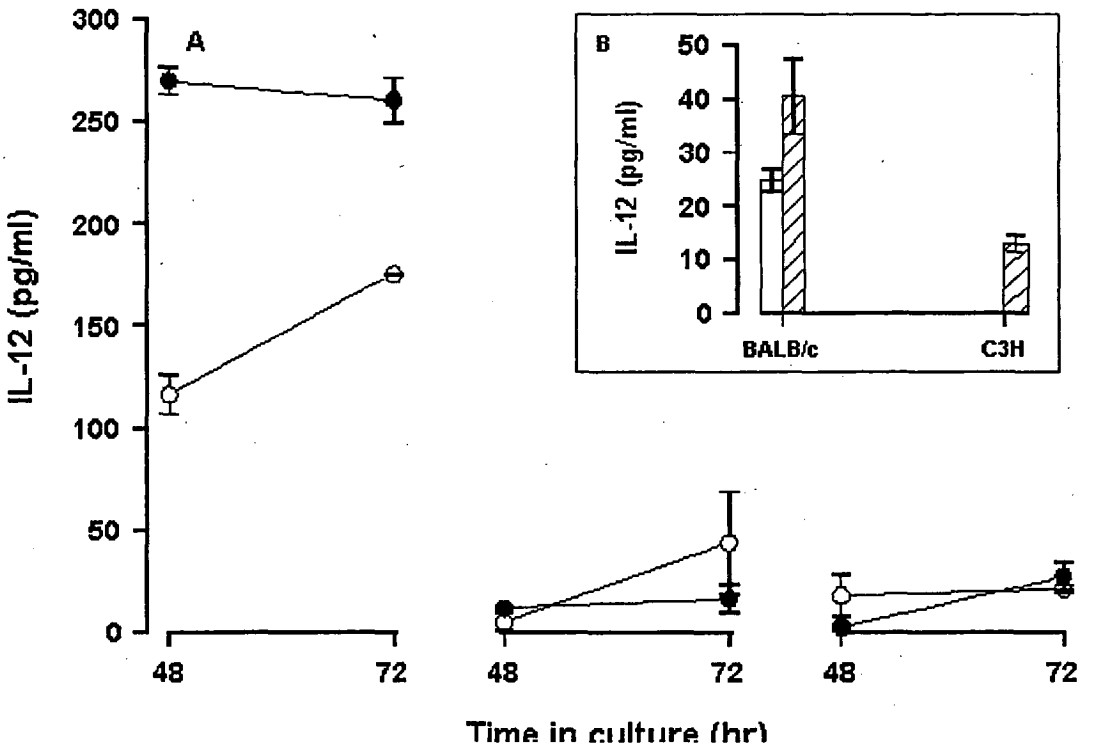


Fig 3.14 *L.major* PM upregulated the production of IL-12p40 by DC. **A.** CD11c$^+$ DC (left panel), CD11c$^-$ DC-depleted LDC (centre panel) and adherent macrophages (right panel) were isolated from BALB/c mice and cultured for 48 or 72 hrs in medium alone (•) or with PM at a DC:PM ratio of 1:5 (o). **B.** CD11c$^+$ DC were isolated from the spleens of BALB/c and C3H mice and cultured for 48hrs in medium alone (□) or with PM at a DC:PM ratio of 1:5 (□shaded). All culture supernatants were filtered and assayed for IL-12p40 by ELISA.

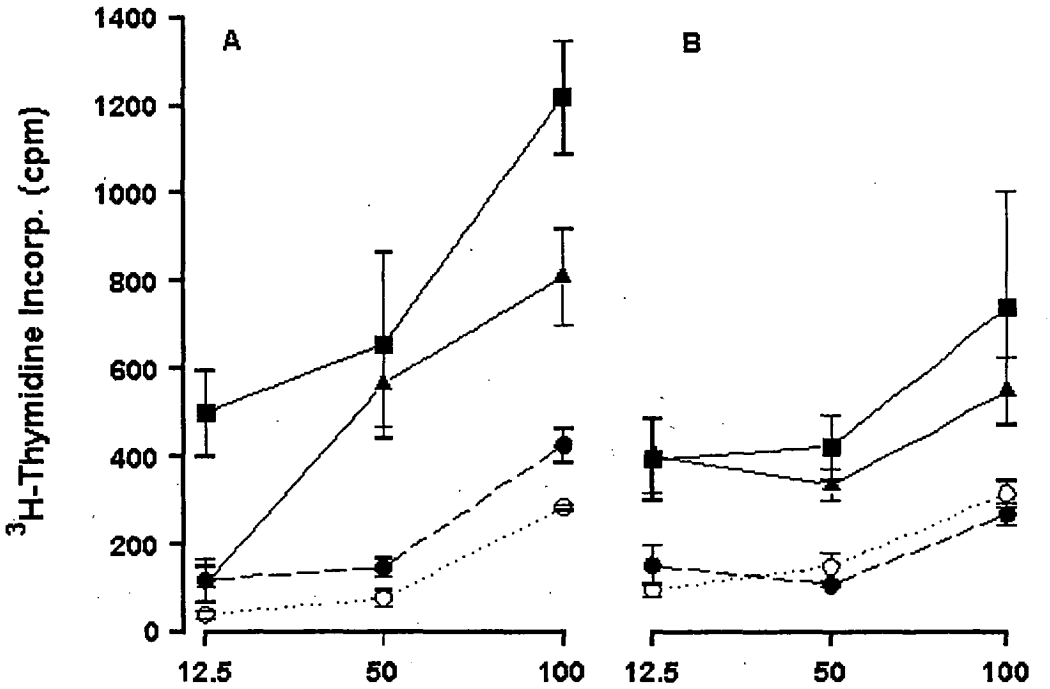


Fig 3.15 DC pulsed with *L.major* PM stimulated proliferative responses in naive T cells obtained from both BALB/c and C3H mice. 2000 DC pulsed with *L.major* antigens were incubated, in hanging-drop cultures, with syngeneic LNC (12,500 - 100, 000 per well) for 3 days and proliferation measured by ^{3}H-Thymidine incorporation. (O····O) LNC alone, (●---●) LNC plus control DC, (▲——▲) LNC plus DC pulsed with PM antigen, (■—■) DC pulsed with PM culture supernatant. (A) BALB/c mice (B) C3H mice. The graph represents data from more than 10 separate experiments.

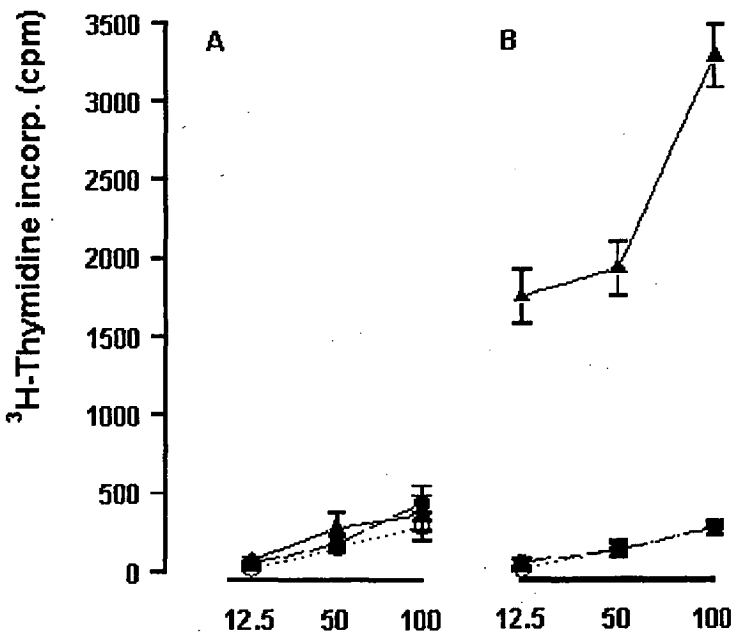


Fig 3.16 DC, but not macrophages pulsed with *L.major* PM stimulated primary T cell responses. 2000 adherent spleen macrophages (A) or splenic DC (B) were pulsed with *L.major* PM for 24 hours and incubated, in hanging-drop cultures, with syngeneic LNC (12,500 - 100, 000 per well) for 3 days and proliferation measured by ^{3}H-Thymidine incorporation. (■- - -■) LNC alone, (●·····●) LNC plus stimulator cells cultured in medium alone, (▲——▲) LNC plus PM-pulsed stimulator cells. The graph represents data from more than five separate experiments.

was confirmed by trypan blue exclusion as outlined in chapter 2. The data shown is representative of more than five experiments.

3.3.2 Anti-IL-12 and primary T cell responses to *L.major* PM

To investigate the role IL-12 played in T cell proliferation and activation in this in vitro system, anti-IL-12 mAb was added to the hanging drop cultures. The presence of anti-IL-12 did not reduce T cell proliferative responses in either resistant or susceptible strains of mice (Fig 3.17). The effect of anti-IL-12 on the cytokine production of the T cells is discussed below. The data represents three experiments.

3.3.3 NLDC-145 blockade and the primary T cell responses to *L.major*

The impact of blocking DC receptors, DEC 205, with NLDC-145, on the uptake of *L.major* PM was investigated with light microscopic analysis. Despite varying doses of NLDC-145 mAb addition to the co-cultures of DC and PM, no reduction in the rate of internalisation was observed when compared with controls. The rate of uptake in the control group was approximately 25% at 24 hours with an average of two parasites per DC (Fig 3.2).

Similarly, treatment of DC with NLDC-145 prior to *L.major* PM exposure and subsequent co-culture in lymphocyte proliferation assays did not impair the stimulatory capacity of *L.major* PM pulsed DC to stimulate a primary immune response in either resistant or susceptible strain of mouse (Fig 3.18).

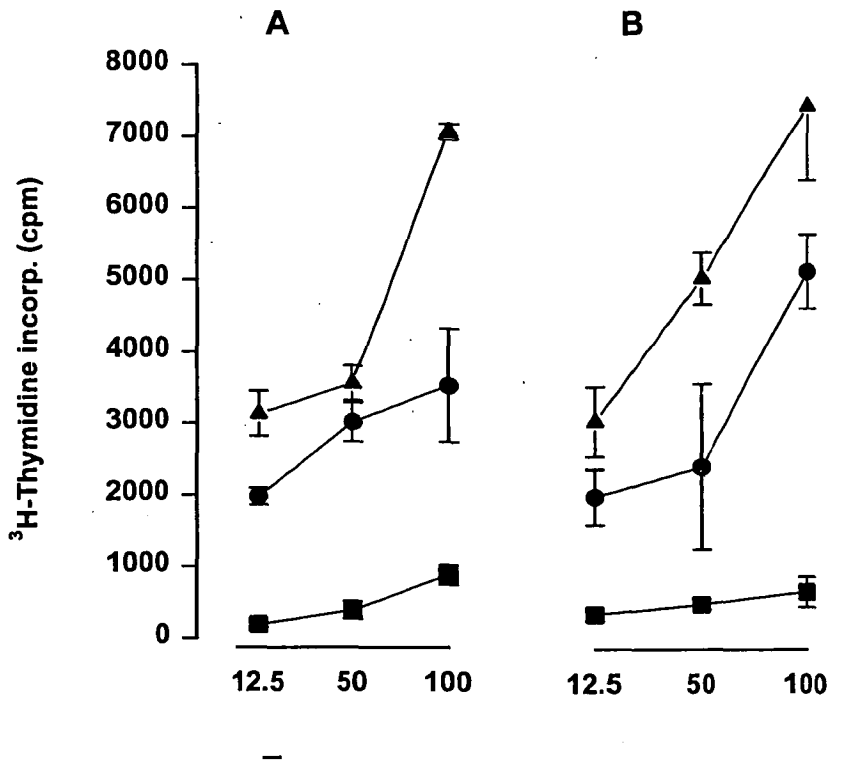


Fig 3.17 The proliferative responses in naive T cells obtained from C3H mice was not affected by the presence of anti-IL-12. (A) 4000 DC pulsed with *L.major* PM were incubated in hanging-drop cultures with syngeneic LNC (12,500 - 100, 000 per well) either alone or (B) with the addition of anti-IL-12 to the culture for 3 days and proliferation measured by ^{3}H-Thymidine incorporation. (■—■) LNC alone, (●—●) LNC plus LDC, (▲—▲) LNC plus DC pulsed with PM antigen. The graph represents data from three separate experiments.

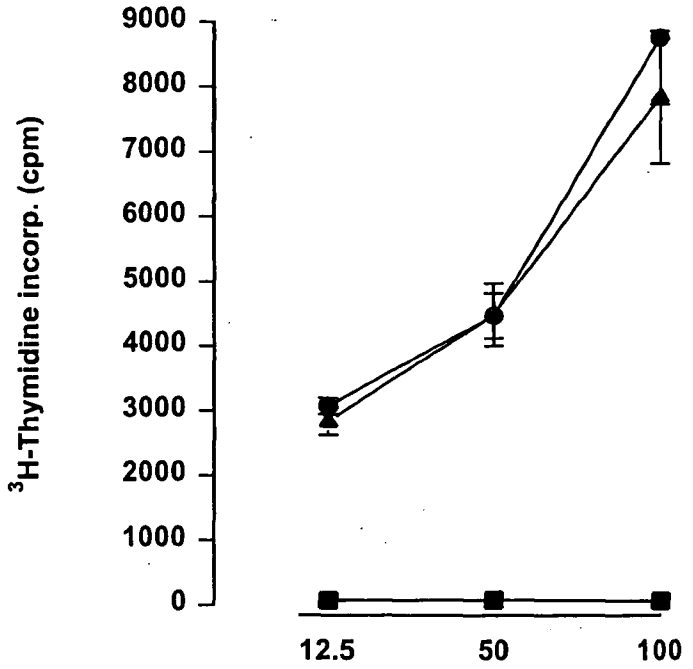


Fig 3.18 The proliferative responses in naive T cells obtained from BALB/c mice was not affected by the presence of NLDC-145 blocking. In 3 day hanging drop cultures 2000 DC pulsed with *L.major* PM were incubated with syngeneic LNC (12,500 - 100, 000 per well) with either prior NLDC-145 treatment of DC (●——●) or with isotype control DC treatment (▲——▲) and proliferation measured by ^{3}H-Thymidine incorporation. (■——■) LNC alone.

3.3.4 Pronase treated supernatant

DC were shown to have the capacity to stimulate primary T cell proliferative responses to pronase treated supernatant from stationary phase *L.major* culture. As the supernatant was treated with pronase, the assumption was made that only non-peptide antigen was present in the treated supernatant. No reduction in the degree of lymphocyte proliferation was observed when treated supernatant was applied to the cultures compared with control supernatant. This suggests that non-peptide antigen may be the major component of the supernatant. These results were repeated in two experiments.

3.4 Secreted Cytokines in the Primary Immune Response

Supernatants aspirated from three day hanging drop cultures where *L.major* PM exposed DC stimulated primary immune responses (Fig 3.15) were analysed by ELISA to quantify the level of IFN-γ, IL-4 and IL-12 produced in the co-cultures.

3.4.1 IFN–γ

IFN–γ was produced in low levels by LNC co-cultured with syngeneic DC in both susceptible and resistant mice after three days in culture. The level of IFN-γ production dramatically increased in the same culture when DC had been previously exposed to *L.major* PM (Fig 3.20). The greater increase in IFN-γ elaboration in the resistant strain, C3H, occurred consistently in three repeated experiments, but was apparent only as a trend, not reaching statistical significance.

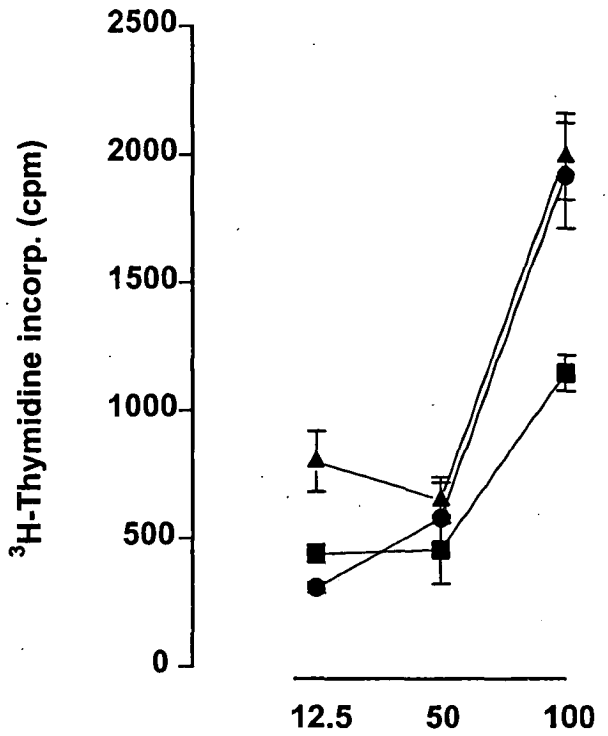


Fig 3.19 The capacity for DC to stimulate primary T cell proliferation when pulsed with stationary phase *L.major* supernatant was not reduced when the supernatant had been treated with pronase for 48 hours. In 3 day hanging drop cultures 2000 DC from BALB/c mice were pulsed with either control *L.major* supernatant (●——●) or pronase treated supernatant (▲——▲) and incubated with syngeneic LNC (12,500 - 100, 000 per well). Lymphocyte proliferation was measured by ^{3}H-thymidine incorporation . (■——■) LNC alone. These data were reproduced in several experiments.

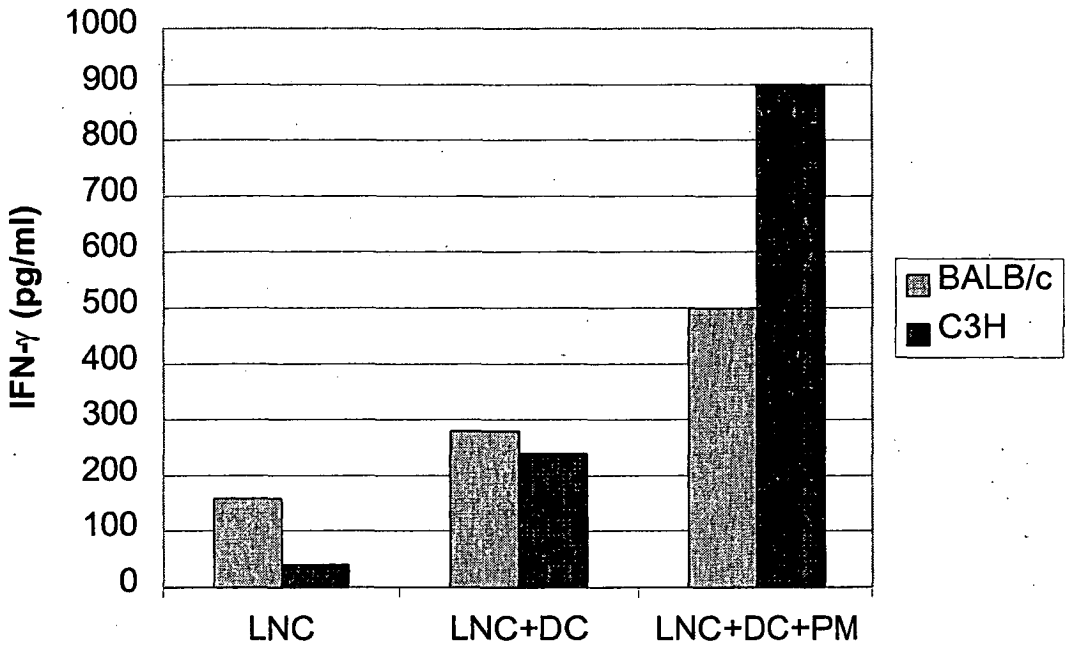


Fig 3.20 IFN-γ production was enhanced in 3 day lymphocyte proliferation assays in both BALB/c and C3H mice in the presence of *L.major* PM. In hanging drop cultures, LNC (12,500 - 100, 000 per well) were cultured alone, with 2000 DC or with 2000 *L.major*- pulsed DC. A low level of background IFN-γ production in the supernatants from LNC alone and LNC incubated with DC (LNC+DC) was dramatically increased with addition of PM to the culture (LNC+DC+PM). There was a trend for the resistant murine strain to produce higher levels of IFN-γ.

Furthermore, the IFN-γ response to *L.major* PM pulsed DC in the primary immune response was shown to be partly IL-12 dependent. The level of IFN-γ production was reduced but not completely inhibited by the addition of anti-IL-12 to the hanging drop assays (Fig 3.21). Interestingly, anti-IL-12 mAb suppressed the production of IFN-γ in the three day cultures but had no impact on the lymphocyte proliferation observed in the same primary immune response (Fig 3.17).

3.4.2 IL-4

IL-4 was barely elevated in the supernatants of LNC alone, LNC plus DC and LNC plus DC pulsed with *L.major* PM in susceptible as well as resistant mice after three days in culture. None of the supernatants in repeated experiments had a mean absorbance above the level which corresponded with an IL-4 concentration of 5pg/ml, the lower limit of detection in these assays, thus indicating negligible IL-4 production in this in vitro model. [3]

3.4.3 IL-12

Exposure of DC to *L.major* PM up-regulated the production of IL-12 p40 in the three day culture (Fig 3.21) corresponding with observed lymphocyte proliferation (Fig 3.15) culture. Additionally there was a trend for greater IL-12 p40 production in the resistant strain, C3H, compared to BALB/c mice, but was not significant in two repeated experiments.

[3] Negligible IL-4 levels is notoriously difficult to detect in part because it is consumed as it is produced. Using anti-IL-4 receptor antibodies may sometimes alleviate this problem but were not used in our experiments.

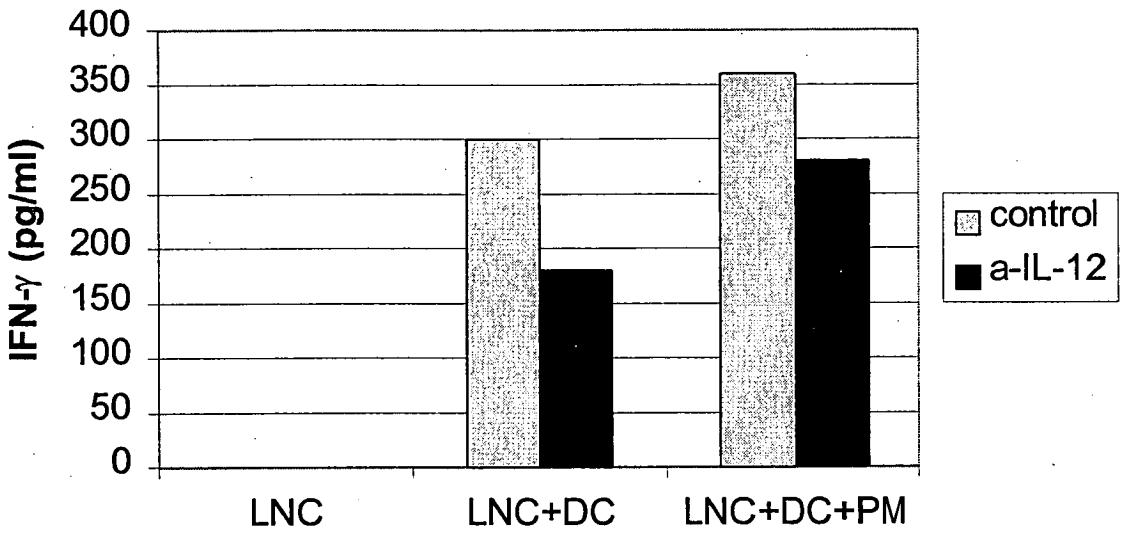


Fig 3.21 The addition of anti-IL-12 mAb to three day lymphocyte proliferation assays reduced the level of IFN-γ produced by LNC cultured with *L.major* PM exposed DC compared to controls.

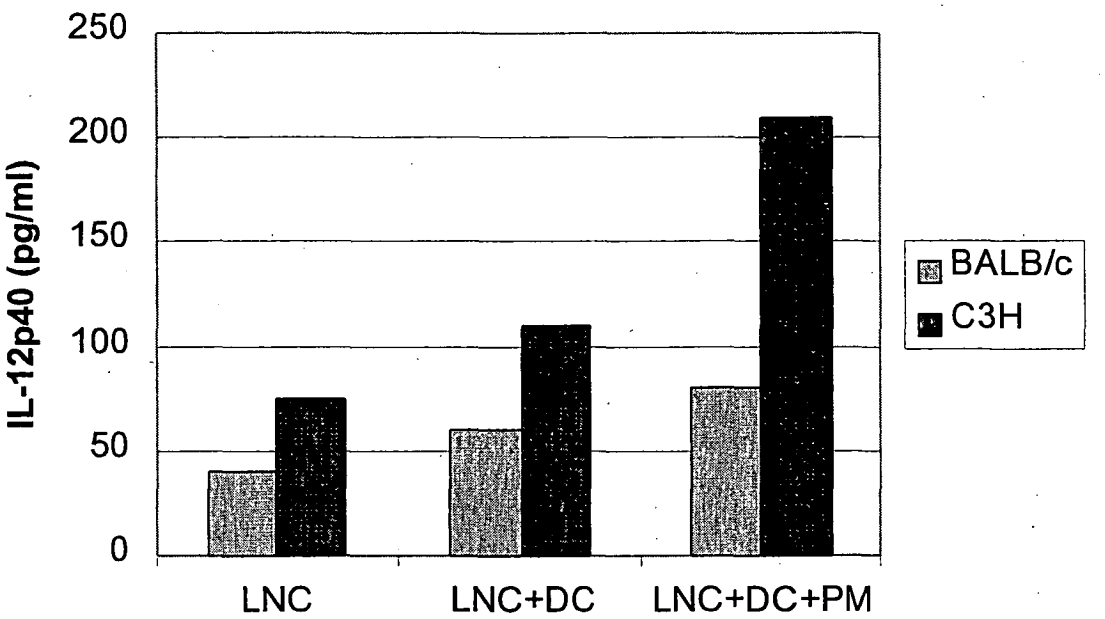


Fig 3.22 IL-12p40 production is increased in 3 day lymphocyte proliferation assays in both BALB/c and C3H mice in the presence of *L.major* PM. In hanging drop cultures, LNC (12,500 - 100, 000 per well) were cultured alone, with 2000 DC or with 2000 *L.major*- pulsed DC. A low level of spontaneous production of IL-12p40 in LNC alone and LNC+DC was increased when the DC had been exposed to *L.major* PM. There was a trend for C3H to produce a higher level of IL-12p40 compared to BALB/c. These results were repeated in 2 experiments.

The same pattern of IL-12p40 production also corresponds with the rise in IFN-γ levels in the same supernatants as outlined above (Fig 3.20).

CHAPTER 4

DISCUSSION

DISCUSSION

4.1 The In Vitro Murine Model

A major component of this thesis was the establishment of an in vitro murine model which could be used to analyse the role of DC in the primary immune response to *L.major* PM. A murine model was chosen due to the existence of resistant and susceptible strains which could be used to investigate whether DC were involved in determinants of disease susceptibility or resistance. To elucidate the mechanism that leads to selective priming of Th1 and Th2 cells, the factors that cause naïve T cells to differentiate down a Th1 or Th2 pathway needed to be determined. Due to the very low frequency of a given T-cell antigen specificity in naïve experimental animals, there is an expectation that LNC from a naïve animal will not respond to stimulation with antigen in vitro. This has caused most investigators to analyse immune responses in intact animals. As the priming of CD4 cells is likely to be multifactorial, defining all of these factors in vivo is difficult. Hence an in vitro model was established.

Titus and colleagues also developed an in vitro model using susceptible BALB/c and resistant CBA mice (Soares, et al 1997; Shankar and Titus 1993). With their model, cytokine patterns were analysed in the supernatants of naïve "spleen cells" stimulated with *L.major* PM in vitro for seven days. Using their in vitro model they were able to define different cytokine patterns corresponding with susceptibility and resistance, however, the APC population in their studies was not identified. Their work is discussed in more detail below.

An alternative method of investigating primary immune responses is to employ TCR transgenic mice which are genetically manipulated such that over 90% of their T cells recognise one epitope of a single model antigen. Transgenic mice have been used extensively in the study of immune responses to leishmaniasis (Kamijo, et al 1993; Dalton, et al 1993; Noben-Trauth, et al 1996; Kopf, et al 1996). However this system has several limitations. *L.major* is a complex dividing host of antigens which profoundly effects APC function in itself, such as the inactivation of macrophages by the PM stage as described above. Model antigens do not have these effects on APCs and furthermore they are internalised in endosomes, whereas *L.major* reside in phagolysosomes. Cytokines often need to be added to these systems for Th commitment to occur (Macatonia, et al 1995). From the outset, these mice have been significantly immunologically altered by the nature of their singular TCR specificity which would have a probable but unquantifiable impact on other cells intimately involved with the primary immune response.

Generating significant numbers of DC to perform in vitro studies has been addressed with derivation of DC from stem cells of mouse bone marrow with a combination of cytokines. However for our purposes, bone marrow derived DC have already been influenced by exposure to cytokines and are therefore a further step removed from the true naïve experimental situation. Furthermore, functionally distinct DC subsets can be generated in these cultures by subtle manipulation of cytokines (Caux, et al 1997) which further complicates investigating APC function.

Systems to analyse primary T cell responses in vitro may prove valuable in assessing the immunogenicity of parasite components for use in potential vaccines. This study

has shown that the murine model developed here is successful in producing reliable and reproducible results. Furthermore, this study demonstrated for the first time that DC of both susceptible and resistant strains of mice were able to internalise *L.major* PM, produce IL-12 in response and stimulate naïve T cell proliferation with cytokine responses favouring Th1 development. There were some limitations to this model and they are described in the context of the discussion of results which follows.

4.2 DC internalisation of *L.major* PM

This investigation demonstrated for the first time that murine DC take up *L.major* PM. With electron microscopy, PM could be seen within the DC, some within phagosomes. A number of observations suggest that the fate of PM within DC is distinct from that within macrophages. DC internalised small number of PM which did not reside within classic PV and did not differentiate into AM. In contrast, under identical conditions, macrophages contained a large numbers of parasites, including AM, within vacuoles. In macrophages the PV have been characterised for several *Leishmania* species with identification of specific cathepsins, hydrolases and membrane proteins (Lang, et al 1994). The DC phagosomes in which PM were seen will require further characterisation.

Experiments in which DC were incubated with fluorescence-labelled parasites indicated a higher frequency of uptake of PM than was suggested by the presence of intact PM within DC in electron microscopy experiments (80% vs 30%). This may reflect rapid degradation of PM within DC, an interpretation which is supported by the presence of PM-derived debris seen within DC (Fig 3.6). This would be in

keeping with the role of DC as professional APC, capable of rapid antigen uptake, processing and presentation to T cells upon reaching regional lymph nodes.

Internalisation of PM by DC probably occurs via macropinocytosis or mannose-like receptor or as yet other unidentified receptor uptake. The protective mechanism of utilisation of CR1 and CR3 macrophage receptors for PM and AM uptake, which prevents triggering of a destructive respiratory burst, is unlikely to be operating in the DC studied here. Interestingly the small numbers of AM which persist intracellularly in LC for prolonged periods also appear to use CR3 for their uptake (Blank, et al 1993).

LC, the DC of the epidermis, have been reported to be parasitised by *L.major* AM (Moll, et al 1993) but not PM (Locksley, et al 1988). LC differ from DC in the dermis and other tissues, particularly with respect to some cell surface molecules, level of maturity and capacity for specific antigen uptake (Caux, et al 1997; Geissmann, et al 1998). Thus, spleen derived DC may represent a population more akin to dermal DC, explaining the difference in uptake compared to LC. The conventional view of DC maturation is that antigen processing is most efficient in immature cells, particularly those that reside in external tissues such as LC (Shuler and Steinman 1985; Romani, et al 1989; Kampgen, et al 1991). These cells are not effective in clustering with and activating T cells, but they become so with maturation when they concomitantly lose their ability to process antigen. However, the reality is probably not so clear cut. DC from the afferent lymph maintain the capacity to process antigens even after 'maturation' in culture (Liu and MacPherson 1995). Other researchers have demonstrated that spleen derived DC, after 16 hours

maturation on plastic, are very efficient at antigen uptake and processing as well as antigen presentation (Coates, et al 1996). Our results also argue against the view that the capacity to process antigens is lost as DC mature and become efficient at clustering and stimulating T cells.

Recently published work has reported DC internalisation of *L.major* PM in a human in vitro system (Marovich, et al 2000). They cite a high rate of internalisation of intact PM (from 38% at day 1 to 70% at 72 hours) by DC and claim doubling of intracellular parasite number from 3.3 to 6.2 organisms per cell by the third day. The organisms have the appearance of intracellular amastigotes in their published photomicrograph suggesting that DC support intracellular parasite survival and replication. This was not characterised or discussed further. Opsonisation of *L.major* PM with 5% normal human serum before infection of DC may have increased PM uptake in Marovich's studies. It has been recently recognised that *L.major* PM bind to components of human serum, particularly IgM to facilitate subsequent invasion of neutrophils and macrophages via complement receptor CR1. This process, however, does not seem to be present in animal models of disease (Dominguez and Torano 1999). In the mouse model, opsonisation with 5% normal mouse serum has not influenced uptake of *L.major* PM in other work (von Stebut, et al 1998).

The DC used in Marovich's work were derived from PBMC using recombinant IL-4 and GM-CSF with a standardised protocol (Sallustro and Lanzavecchia 1994), and were thus distinctly different from spleen derived DC. DC are heterogeneous, even within the spleen (Vremec and Shortman 1997). Therefore different DC are likely to differ in their interaction with *L.major*. The myeloid-derived DC may represent a

more pure population of a particular subtype of DC capable of phagocytosis. In particular, their DC population probably comprised less mature DC than the splenic DC used in our system, as suggested by the lower expression of CD86 on the former, and therefore possess a greater capacity for antigen capture. Although $CD14^+$ cells are excluded from the population of DC by negative selection, Marovich's DC may have possessed some macrophage-like characteristics. It is not clear why CD83, a specific human DC marker was not used to positively identify DC, particularly infected DC, in their work.

Avid uptake by DC of another intracellular pathogen, *Histoplasma capsulatum* yeasts was recently described by Gildea and co-workers (Gildea, et al 2001). They found that DC rapidly killed the yeasts, unlike macrophages which support pathogen replication, similar to our observations with *L.major*. Furthermore they demonstrated uptake of the yeast via an alternative DC receptor, very late antigen-5 (VLA-5), even though the DC possessed CD18, a known receptor used for the uptake of *Histoplasma capsulatum* by macrophages. The difference in receptor usage may account for the ability of DC to inhibit the intracellular growth of *Histoplasma capsulatum,* possibly by triggering a different intracellular signalling cascade. A similar situation is probably occurring in our study and, as discussed above, is an area yet to be explored with the uptake of *L.major* by DC.

Our findings were contradicted by another study which showed DC to be incapable of taking up *L.major* PM (von Stebut, et al 1998). Again, a different murine model was used, where the source of DC was murine fetal skin-derived (FSDDC). Immature FSDDC were propagated in a GM-CSF and M-CSF supplemented media

for two weeks and DC aggregates isolated by centrifugation and dissociated by trypsin/EDTA. Surface antigen expression indicated a different subgroup of DC, which additionally had been heavily pre-treated with cytokines and may have lost as yet undefined surface membrane properties for the internalisation of some antigens, including *L.major* PM.

Enough evidence now exists to refute earlier observations that dermal macrophages are the only site of initiation of *Leishmania* infection (Locksley, et al 1988).. It would appear that DC derived from the spleen in this model were at an optimal stage for antigen uptake and presentation as supported by other workers (Coates, et al 1996). Given that the results generated from this study, as well as the results of more recent work of other researchers, confirms the capacity for DC to internalise *L.major* PM, the mechanisms of uptake and pathways utilised in PM processing now needs to be further characterised.

The close apposition of infected DC with macrophages strongly suggest intercellular cross-talk and possibly priming and activation of macrophages which could be further investigated by analysing the costimulatory, adhesion and MHC antigens of such macrophages. Cross-talk or cross-priming has been observed in other systems. DC loaded with apoptotic bodies derived from macrophages infected with influenza virus can stimulate the proliferation of influenza specific T cells and the generation of class I MHC-restricted influenza-specific $CD8^+$ cytotoxic lymphocyte reactions (Albert, et al 1998). In the aqueous humour outflow tract of the rat eye, DC and macrophages have been observed in intimate

association with mast cells, the interactions of which may play a role in anterior chamber immune dysfunction (Ioffreda, et al 1992).

Evidence is mounting to suggest that mast cells and skin DC interact. Mast cell-derived TNF–α has been shown in vitro to upregulate certain integrins on LC in human tissue (Ioffreda, et al 1993). Fractalkine, is a membrane-bound CX3C chemokine constitutively expressed on DC as well as skin endothelial cells. The fractalkine receptor, CX3CR1 recently identified on murine mast cells has been shown to effectively mediate chemotaxis without degranulation, suggesting a possible role for specific skin cells, including DC, in tissue-specific homing of mast cells (Papadopoulos, et al 2000). No work to date has investigated the role of mast cells in the pathogenesis of leishmaniasis, an area which may have great potential. This area will be pursued further by the author of this thesis in the research facility she is associated with in her current position where mast cell research expertise is established, using the principles involved in the in vitro model described here.

4.3 DEC-205

The role of DEC-205, a DC mannose-like receptor in the uptake of *L.major* PM was investigated. Blocking experiments using the mAb, NLDC-145, to block DEC-205, had no impact on the uptake of *L.major* PM or the capacity of *L.major*-exposed DC to stimulate lymphocyte proliferation. However, DEC-205 is rapidly regenerated back to the cell surface membrane (Jiang, et al 1995) so there is a possibility that while DEC-205 does not play a predominant role in the internalisation of antigen, it may still be a carrier to assist in PM uptake. Another possibility is that the epitope

for NLDC binding is distinct from the ligand binding site and therefore does not compete. These observations also enabled studies to continue using purification of DC by NLDC-145 selection without concern that DC interaction with *L.major* may be affected by NLDC-145 treatment of DC.

4.4 Costimulatory molecule expression by *L.major* exposed DC

The splenic DC used in this work maximally expressed costimulatory molecules, CD40, CD80 and CD86, adhesion molecules CD44, CD54 and MHC Class I and II antigens, which suggests they were a more mature population of DC. This level of surface molecule expression was shown to be unaffected by uptake of *L.major* antigen. Other workers have described conflicting results (Marovich, et al 2000). Their specific analysis of *L.major* PM infected cells, using LPG labelling of DC to indicate infection, found that HLA-DR, CD86 and to a much lesser extent, CD40 was upregulated on LPG+ve DC compared to controls. Again, this is consistent with the starting DC population being less mature, therefore greater differences may arise from incubation of *L.major* PM with DC at an earlier stage of maturation. Nevertheless in both systems *L.major* PM exposure did not inhibit maximal costimulatory, adhesion and MHC molecule expression an important consideration for future therapeutic studies with DC.

4.5 DC production of IL-12

Following incubation with PM, spleen DC were observed to up-regulate the production of IL-12p40. The cytokine was unlikely to be derived from

contaminating macrophages since it was observed in highly purified DC preparations and macrophage-enriched spleen adherent cells did not produce IL-12p40. Furthermore, *L.major* PM have previously been reported to inhibit IL-12 production by macrophages (Carrera, et al 1996; Reiner, et al 1994). Recent in vivo experiments in models of *Toxoplasma gondii* (Reis E Sousa, et al 1997) and *L.donovani* (Gorak, et al 1998) infection suggest that DC but not macrophages are the predominant source of IL-12 under conditions in which macrophages are not pre-primed by exposure to inflammatory stimuli. In our experiments with *L.major*, IL-12 release appeared to be a direct effect of the parasite on DC and did not require the presence of other cell types. Early in infection, direct stimulation of IL-12 release may be more important than pathways dependent on CD40 ligand expressed on activated T cells (Macatonia, et al 1995; Cella, et al 1996). We observed production of IL-12p40 in cultures of PM-stimulated DC from both BALB/c and C3H mice. Collectively these data suggest that DC are the likely source of IL-12p40 which has been observed in lymph nodes of both resistant and susceptible mouse strains following infection with *L.major* (Scharton-Kersten, et al 1995).

IL-12p70 needs to be measured in these assays and the tests to do this became available only as the study period was drawing to a close. Recently, Marovich and co-workers used a very sensitive intracellular cytokine assay to determine the level of IL-12p70 in DC and indeed found an upregulation of IL-12p70 occurring in *L.major* infected DC (Marovich, et al 2000). Interestingly they also described a requirement for CD40 ligation of the DC to stimulate IL-12p70 production.

There are at least two subpopulations of $CD11c^+$ DC in the spleen (Vremec and Shortman 1997; Leenen, et al 1998) NLDC-145^+ $CD8^+$ interdigitating cells are localised primarily in the T cell areas of white pulp whereas DC in marginal zones are NLDC-145^- $CD8\alpha^-$. The observation that the NLDC-145^+ DC subpopulation has been reported to be the predominant producer of IL-12 (Pulendran, et al 1997) supports our findings of upregulation of IL-12 by NLDC-145^+ selected DC upon exposure to *L.major* PM. Which subgroup of the $CD11c^+$ DC were the predominant IL-12 producers was not determined. The NLDC-145^+ $CD11c^+$ DC were only a minority and what proportion of the IL-12 was produced by this sub-fraction was not analysed. Whether IL-12 was also released by other DC subsets remains to be determined. Again, DC from both BALB/c and C3H were capable of upregulating IL-12 in response to *L.major* PM exposure.

The enhanced IL-12 production in the lymphocyte proliferation assays with DC had been pulsed with *L.major* confirms the presence of this cytokine in the primary T cell response to *L.major*. The trend for the resistant mouse strain to produce more IL-12 than the susceptible mice was an intriguing observation but needs to be confirmed in repeated experiments and also with other resistant murine strains.

4.6 Primary immune responses

DC, but not macrophages, exposed to *L.major* PM or PM culture supernatant were able to stimulate proliferative responses in lymph node cells from naïve mice of both BALB/c and C3H strains. Titus and colleagues also described stimulation of naïve T cells by *L.major* antigens in their primary in vitro system (Soares, et al 1997).

However, the APC were not identified in the 'splenocyte' population used for restimulation of T cell blasts. They also described a requirement for whole viable parasites which was not necessary in our studies with isolated DC. It remains to be determined whether the stimulatory material in supernatants is actively secreted by the parasite or is derived from the organisms dying in culture

We found no difference in the way DC interact with *L.major* PM between resistant and susceptible strains of mice. Specifically, both susceptible and resistant strains were equally capable of internalising *L.major* antigen, producing IL-12 in response and stimulating T cell proliferation with IFN-γ production. Thus DC have the potential to present *L.major* antigen and induce Th1 development in the early stages of infection.

It appears that other factors outside the DC-antigen-T cell interaction may determine the phenotypic pattern of resistance or susceptibility to leishmaniasis. T cell responsiveness, in particular the capacity for T cell upregulation of its IL-12 receptor, may be one mechanism which contributes to disease susceptibility or resistance (Guler, et al 1996). External factors in vivo may suppress DC function. Splenic DC has been observed two months post-infection with *L.donovani* in susceptible mice (Basu, et al 2000). Disease progression in these studies is associated with impaired DC production of IL-12 and reduced MHC class II antigen expression and ability to stimulate IFN-γ production by *Leishmania* antigen-primed T cells. IL-10 which is produced in significant levels in *L.donovani* infection is capable of converting immature DC into tolerogenic APC and down-regulate HLA-DR (Steinbrink, et al 1997). Prostaglandins which are produced in copious quantities by *Leishmania* can

also impair the ability of maturing DC to produce IL-12 and therefore promote Th2 responses (Kalinski, et al 1997).

Thus in using this in vitro system it appears that DC have an inherent capacity to internalise antigen and potentially drive a Th1 response. This system therefore remains a useful tool for the testing of antigens which may promote development of Th1 responses and could therefore be used in vaccination and therapeutic research. External factors and how they influence DC function need to be further investigated to optimise the antigen presenting capacity of DC in vivo.

4.7 IFN-γ and IL-4

IFN-γ was present at low levels in the supernatants from the lymphocyte proliferation assays in both susceptible and resistant strains of mice. Stimulation with *L.major* PM-exposed DC triggered a dramatic increase in IFN-γ production within the cultures, consistent with the proliferative T cell response observed. Furthermore the IFN-γ production was partially inhibited by neutralising IL-12, indicating that IFN-γ upregulation is at least a partially IL-12 dependent process.

IL-4 on the other hand was barely detectable, an observation found in other primary in vitro assay systems (Soares, et al 1997). Whether this finding is a true reflection of the production of IL-4 or is due to rapid consumption of the cytokine could be addressed by using anti-IL-4 receptor antibodies. Alternatively, there may be another source of IL-4 which has not yet been elucidated. Mast cells are potent producers of IL-4 and IL-5, bear MHC class II antigen and also reside in the dermis. Whether mast

cells play a role in the primary immune response to *L.major* is an intriguing question that warrants further investigation. Yet other cytokines which have been reported to play a role in the initial response to *L.major* infection, such as IL-10 and TGF-β in particular also warrant further investigation in this primary in vitro system.

Recent work has described the effect of specific splenic DC subsets on the regulation of T cell cytokine production (Kronin, et al 2000). Specifically, $CD8a^-$ DC induced a much higher production of IL-3, IFN-g, GM-CSF and IL-2 in primary CD8 T cells and of IL-2, IL-3 and IL-10 production in primary CD4 T cells compared with $CD8^+$ splenic DC. Interestingly, even when exogenous IL-2 was added to the primary cultures the higher level of cytokine production was maintained. Thus evidence that the general control of T cell cytokine production by splenic DC involves factors additional to those that govern activation and proliferation of T cells is now emerging. As with our study, these authors reiterate that many other factors which influence T cell cytokine production may be operating in vivo. However, the tight rosette-like clustering of DC and primary T cells which is required to initiate an immune response may be quite well reflected by a reductionist in vitro which only examines DC:T cell interactions. The results of further studies quantifying the T cell subsets in the nylon wool purified T cells and looking specifically at DC:T cell interactionsin this model will be of great interest.

4.8 Non-peptide antigen presentation

Of particular interest was the observation that DC could stimulate T cell proliferation after exposure to *L.major* stationary phase supernatant. Lymphocyte proliferation

was unaffected by elimination of the peptide component from the supernatant, thus suggesting that non-peptide antigen from *L.major* could be presented by DC to T cells to induce a proliferative response. The non-MHC encoded CD1 family recently emerged as an antigen-presenting system that is entirely distinct from either MHC class I, class II or related class I-like and class II-like molecules (Beckman, et al 1994). CD1 molecules, present on DC are capable of presenting non-protein antigens to T cells, unlike the MHC system which presents exclusively peptides. The role of CD1 molecules is yet to be fully elucidated. Evidence to date suggests they may have a specific immunological function which could play a pivotal role in inducing a Th2 response via ligation of a specific conserved subset of $NK1.1^+$ T cells which are early producers of IL-4 (Bradley, et al 1993). Thus either alone or at least in concert with MHC antigen presentation, CD1 molecules may play a significant role in stimulating primary immune responses and further characterisation of their role in this system would be of particular interest.

The immunostimulatory antigen in the supernatant may be a component of LPG. Due to time restraints, further analysis of the treated supernatant, with high performance liquid chromatography to establish the nature of its constituents was not performed. It would also be of importance to ensure pronase treated supernatanat was indeed devoid of protein, as discussed earlier, some proteoglycans secreted into the supernatant are notoriously resistant to proteases (Ilg 2000). Fractionating the supernatant into various constituents and using this in vitro system to measure proliferative T cell responses to different fractions would be of interest and could follow on from these preliminary studies.

CHAPTER 5

SUPPLEMENTARY CLINICAL STUDY

SUPPLEMENTARY CLINICAL STUDY

5.1 Introduction

There is a great need for the development of safe, practical and efficacious treatment for cutaneous leishmaniasis (CL) as this disease affects 1-1.5 million people world-wide annually (Desjeux 1996). Treatment for CL became a World Health Organisation/Tropical Disease Research priority in the 1990's. During my laboratory based research into the role of DC in the pathogenesis of leishmaniasis, I had the opportunity to partake in a pilot study trialing a novel nitric oxide based cream for the treatment of CL in Syria, an area hyperendemic for CL.

Based on previous experience with nitirc oxide (NO) and its cidal properties for pathogens (Duncan, et al 1995), Professor Nigel Benjamin, at St Bartholomew's, London theorised about the potential role of NO-releasing creams in the treatment of leishmaniasis. Vanessa Yardley and Simon Croft, at the London School of Hygiene and Tropical Medicine, performed in vitro assays with *Leishmania* infected macrophages and in vivo studies with *L.major* in mice testing the efficacy of a range of creams which would release nitric oxide locally. The author was not involved in these studies. The details of these laboratory based investigations are described in the publication (Davidson, et al 2000) attached in the appendix of this manuscript. The encouraging results from the dose ranging in vitro macrophage studies and the murine studies prompted the initiation of a pilot study by Dr Robert Davidson and the author in collaboration with Professor Hratch Balaban, Department of Dermatology, University of Aleppo, Syria to investigate the clinical efficacy,

tolerability and practicality of NO-releasing creams in the treatment of patients with CL. This work was supported by a Director's Initiative grant from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. The author's role as a co-applicant on the grant was to assist in setting up the study at the University of Aleppo's Dermatology clinics with Dr Robert Davidson and Professor Hratch Balaban and to analyse the results.

5.2 Background

Standard treatment of CL is by local injection of pentavalent antimonials into the lesions, or for more severe forms, systemic pentavalent antimonial injections. Local lesional treatment is painful, may be protracted for weeks or months in recalcitrant lesions and is not always successful. Parenteral therapy has significant side effects (reviewed by Berman 1996). Macrophages infected with *Leishmania* rely on NO production to kill parasites as reviewed in Chapter 1. The mechanism of NO production is via 5 electron oxidation of L-arginine by the inducible enzyme nitric oxide synthase (iNOS). iNOS is inhibited by *Leishmania* both directly and by an inhibitory effect of cytokines regulating iNOS production (Bogdan et al, 1996). iNOS deficient mice are unable to control the spread of *Leishmania* (Diefenbach, et al 1999).

An alternative pathway for NO production is non-enzymatical acidification of nitrite. This produces nitrous acid (HNO_2) which has an acid dissociation constant of 3.2, so that in the presence of a low pH complete conversion will occur, with subsequent decomposition of nitrous acid to various oxides of nitrogen, including NO thus:

$NO_2^- + H^+ \rightarrow HNO_2$

$2HNO \leftrightarrow N_2O_3 + H_2O \leftrightarrow NO + NO_2$

$3HNO_2 \leftrightarrow 2NO + NO_3 + H^+ + H_2O$

It is not clear which reactive nitrogen intermediate is responsible for NO mediated microbial killing in mammalian cells. Indeed, different organisms may be susceptible to different reactive nitrogen species (reviewed by Fang 1997). Acidification of nitrite results in a complex mixture of nitrogen oxides as well as nitrous acid. Nitrous acid, dinitrogen trioxide and nitrogen dioxide are all effective nitrosating agents, that is, NO+ donors (Dykhuizen, et al 1996). In vitro, *L. mexicana* amastigotes are rapidly killed and structurally damaged by the addition of nitrite to their acidified (pH~5.5) culture medium (Reece, et al 1995). The toxicity of the solution correlated with the concentration of a reactive nitrogen intermediate, nitrous acid, and was cidal to *Leishmania* in concentrations of 0.1 to 10 mM.

NO has the property of rapidly diffusing through tissues. When NO encounters superoxide or oxidised haemoglobin it is rapidly oxidised to nitrate, and becomes inert. Topical treatment for CL, in which the parasites are found in the dermis to a depth of 5 mm or more, is problematic because of the very limited penetration of drugs through the skin. As NO is so freely diffusible, we reasoned that it may be possible to apply an acidified nitrite cream over the lesions of CL, and thereby generate enough NO exogenously to kill *Leishmania* without causing damage to host tissues. NO kills *Leishmania* in vitro at concentrations much lower than that which kills tissue cells. NO has a very short half-life, so it is difficult to measure, and

nitrate is often measured as a surrogate for NO production. We could not do this in our experiments because nitrite was a constituent of our mixture.

5.3 Materials and methods.

5.3.1 Drugs and Formulation

The constituents of the NO-producing cream were made up separately. Potassium nitrite (KNO_2), salicylic acid (SAL), ascorbic acid (ASC) (all from Sigma) and potassium chloride (KCl) (Merck) were each added separately to an aqueous base. Due to the short half-life of NO, the nitrite cream and acid containing cream were mixed immediately prior to application.

The manufacturing department of the pharmacy at Northwick Park Hospital made up 150g quantities of constituent creams which were despatched by courier to the study centre in Aleppo, and stored at 4 °C until dispensed.

5.3.2 Tolerability in volunteers

Two of the investigators, including the author of this manuscript, applied various combinations of the creams to the flexor surface of the forearm in circles of 3cm diameter twice daily for 7 days. Erythema and tingling of the underlying skin was noted. The erythema faded within three minutes after removal of the cream, suggesting that NO-mediated vasodilatation rather than an inflammatory reaction had occurred. In short-term use, the creams were non-irritant.

5.3.3 Clinical study

Dr Robert Davidson and the author of this thesis travelled to Aleppo with the study protocol and treatments to set up the pilot study in collaboration with Professor Hratch Balaban. After joint discussion, the trial protocol was established as follows.

Adult patients with parasitologically-confirmed lesions of CL attending the Department of Dermatology, University of Aleppo, Syria, were eligible for enrolment. Patients gave informed consent, and the study was approved by the University ethics committee. Patients were excluded for the following reasons: pregnancy or lactation; any underlying condition which compromised the subject's ability to complete the protocol; underlying heart disease, renal failure, hypertension or any other medical condition requiring regular medication; steroid therapy, HIV infection, or any other cause of immunodeficiency. Lesions were considered unsuitable for NO cream treatment if they were on the face, or over a joint; were greater than 6 cm diameter; had obvious bacterial superinfection or were acutely inflamed or painful.

At the start of treatment, the site and size of lesions were recorded, and the lesion photographed. The details were documented on recording sheet 1 (Fig 5.1, 5.2, 5.3). Trial cream was applied twice daily by the patient. Patients mixed similar amounts of the two constituents with a finger-tip on the lesion, to cover the lesion and about 0.5 cm of skin on all sides of the lesion, once daily after washing and drying the lesion. Where anatomically possible, such as limb lesions, an occlusive dressing was placed over the lesion after applying NO cream. Where multiple lesions were present, all

Recording sheet 1: cutaneous leishmaniasis study

Patient name
date of birth___/____/19____ M/F
address telephone number

Cream to be used:

Has patient read the information sheet? **Y**/N
Has patient (or guardian) given written consent? **Y**/N
Is patient able to attend for 8 weeks? **Y**/N
Is patient excluded from the study? Y/**N**
Has parasitological confirmation been done **Y**/N

Visit	date	measure lesions	give cream	adverse events (specify)	sign
day 0					
1 week					
2 weeks					
3 weeks					
4.weeks					
8 weeks					

At 8 weeks was the response to treatment
Good = all lesions healed or substantially improved
Fair = signs of improvement in some or all lesions, but none healed
Poor = lesions worse or no better

At 8 weeks was the patient's use of treatment
Compliant = used at least 5 times each week; collected all supplies
poorly compliant = used erratically; missed 1 or more supplies
incomplete = patient defaulted during treatment

At 8 weeks was the tolerability of the cream
Good = little or no local irritation; no systemic symptoms
Fair = moderate local irritation ; any systemic symptoms (specify)
Poor = could not continue use because of side effects (specify)

Signed: date:

Fig 5.1 Record sheet 1 for entering patient data

Recording sheet 2: cutaneous leishmaniasis study

Patient name Cream used:

Record site of all lesions as a dot

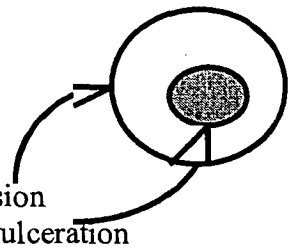
Number 3 or less lesions which will be measured

Indicate when each lesion was first noticed e.g.

- 1 (15/4/96).
- 2 (30/4/96)

Fig 5.2 Record sheet 2 for entering data on patient's lesions

Recording sheet 3: cutaneous leishmaniasis study



Patient name
Measure each lesion:

Cream used:

day 0 date PHOTO	1	2	3
1 week date	1	2	3
2 weeks date	1	2	3
3 weeks date	1	2	3
4.weeks date	1	2	3
8 weeks date PHOTO	1	2	

Fig 5.3 Record sheet 3 for recording data on patient's lesion

were treated with NO cream. At weekly visits, a record was made of lesion size and adverse events, and cream for a further week was issued (Fig 5.1,5.2,5.3). After 8 weeks of treatment, the response to treatment was graded as cured if all ulcers had re-epithelialised and all nodules flattened, improved if signs of improvement in some or all lesions, but not all lesions, or unresponsive if lesions were worse or no better.

5.4 Results

Data was collected by Professor Hratch Balaban and his team as per data sheets (Fig 5.12,5.2,5.3). This data was forwarded to our Department for collation and analysis.

Forty patients with parasitologically-proven CL from Aleppo, Syria were treated with topical nitrite in aqueous cream combined with KCl, ASC, or SAL. There were 22 males and 18 females. The median age was 30 years (range 5-80 years). There were a median of 2.2 lesions (range 1-9) on each patient. The lesions had been present for a median of 3.6 months (range 1-12 months). Several patients reported inflammatory or itching reactions to the creams, which was common with all preparations to some extent. There were no other adverse events.

The response to treatment is summarised in Table 1. Only 11/40 patients (28%) showed improvement and only 5/40 (12%) were cured at 2 months. The maximum possible cure rate at 2 months, if all 6 patients lost to follow-up had been in fact cured, would be 11/40 (28%). The 5 patients who were cured had a similar number of lesions (median 2) as the 35 patients who were not cured and a similar duration of illness (median 4 months).

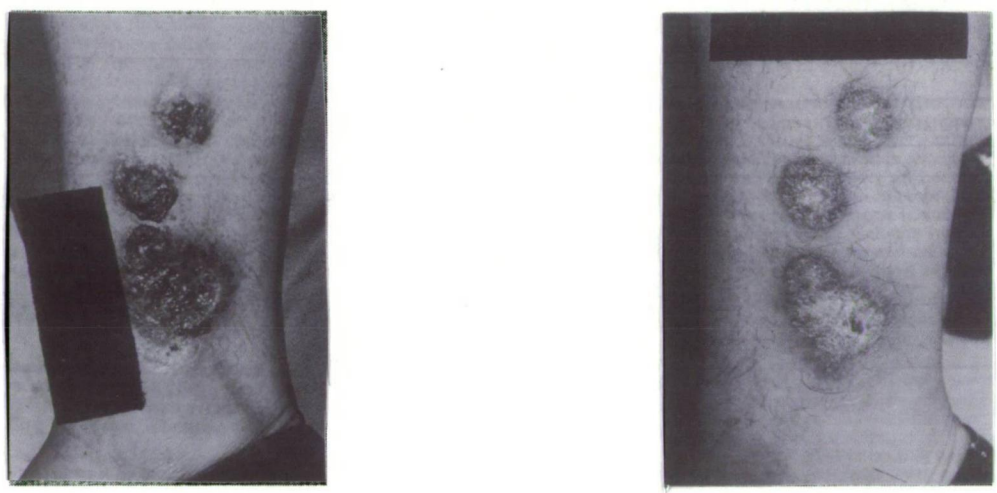
Treatment	**enrolled**	**unresponsive**	**improved**	**cured**	**Lost to follow up**
2%NO_2/2% KCl	4	0	3	1	0
2%NO_2/2% salicylic acid	6	3	2	1	0
5%NO_2/2% salicylic acid	4	4	0	0	0
2%NO_2/2% ascorbic acid	18	8	5	2	3
5%NO_2/5% ascorbic acid	8	3	1	1	3
Total	40	18	11	5	6

Table 5.1 Clinical responses at 8 weeks of 40 Syrian patients with *L. tropica* cutaneous leishmaniasis, who were treated with NO-generating creams for 4 weeks.

A typical case is shown in the photographs (Fig 5.4A and B). A 34 year old lady had 6 nodular lesions on her legs and one ulcerated oozing lesion on the lateral aspect of her right lower leg. The ulceration was 1.5 cm in diameter and had been oozing for nine months. The slit skin smear was positive for *Leishmania*. She was treated with 5%NO_2/5% ASC under occlusion. After 1 week, the ulceration and oozing persisted, and the nodular lesions were noted to have become inflamed. After two weeks of treatment the ulcer had become dry and the nodules less infiltrated. After three weeks of treatment some blisters were noted around the lesions and the treatment was stopped. Application of 5%NO_2/5% ASC to the patient's hand produced a similar reaction at 48 hours, suggesting she had become sensitised to the cream. Treatment was not re-introduced. By eight weeks all the lesions had healed leaving hyperpigmented areas.

5.5 Discussion

The topical treatment of CL is limited by the fact that the infection is located throughout the dermis, as deep as the subcutaneous tissues, and the penetration of an anti-leishmanial compound into a lesion is likely to be minimal. NO is a molecule which could, conceivably, diffuse into the dermis, because of its small size and ability to diffuse through aqueous solutions and lipid membranes. We had hoped to devise a method of acidifying nitrite over the surface of a CL lesion, and that NO might diffuse through the lesion in sufficient concentration to kill *Leishmania* but leave host tissues intact. Whilst nitrite combinations were efficacious in vitro and showed some benefit in murine CL, our clinical study was disappointing in both the low cure rate and the high rate of local, albeit minor, reactions.



A. **B.**

Fig 5.4. A 34 yr old Syrian woman with *L. tropica* cutaneous leishmaniasis. The photographs show 3 of her 6 lesions before (A) and 4 weeks after treatment with 5%NO_2 plus 5% ASC cream (B).

The only topical treatment which has worked in controlled trials in CL was aminosidine 15% in methylbenzethonium chloride applied twice daily for 10 - 30 days. This was effective in Israel in *L. major* CL (El-On, et al 1992) but produced burning and pruritis in 25% of patients and vesicle formation in 15% of patients (Soto, et al 1995). Recently, success was reported using a cream containing the NO donor S-nitroso-N-acetylpenicillamine at a concentration of 200 mmol/L. When applied five times daily for 10 days in an uncontrolled study, all 16 patients with *L. braziliensis* CL in Ecuador were cured, with only mild local reactions to the cream (Lopez-Jaramillo, et al 1998).

There are several possible explanations for the low success rate in our study. Firstly, the species of *Leishmania* which causes CL in Aleppo is *L. tropica* (Ashford, et al 1993) and this may be harder to cure than other forms of CL. *L. tropica* is known to have a slow evolution of lesions and a slow rate of spontaneous healing, with lesions healing in 10-14 months or longer (Bryceson 1996). This sets it apart from *L.major*, *L. braziliensis* and *L. mexicana* and the murine studies were performed with *L.major*. Prior treatment did not exclude the patient from the trial and there was little clinical detail on prior treatment. This would need to be more clearly documented as there may have been a bias in this study towards selecting patients with recalcitrant lesions. More prolonged therapy may also be worth considering, although the local adverse reactions may limit this.

Despite the results presented here, we consider the principle of treating CL with NO releasing creams remains important to continue to assess for efficacy. Other NO-donor compounds such as glyceryl trinitrate may have less local side effects and

should be considered. There are other regions where *L.major* is the predominant cause of CL and with NO releasing therapy could be trialed in those patients. There is enough demand for better CL therapy and encouraging evidence from several studies, including ours, to suggest that formal randomised, placebo-controlled trials with NO releasing treatment should be undertaken.

CHAPTER 6

CONCLUSION AND FUTURE DIRECTIONS

CONCLUSION AND FUTURE DIRECTIONS

The murine in vitro study, which comprises the major part of the manuscript, and the clinical study have contributed to the scientific literature in both pathogenesis and treatment of leishmaniasis.

The results of the laboratory based research have demonstrated that DC play an important role in establishing an early primary T cell response to *L.major* PM. DC internalise *L.major* PM with maximal expression of costimulatory and MHC Class II molecules and in response produce IL-12. Combined with results of the primary stimulation assays these findings suggest that DC from both susceptible and resistant mouse strains are able to stimulate autologous naive T cells when pulsed with PM antigens and provide a cytokine environment predicted to favour the development of a Th1 response. This work combined with the findings of other investigators has established the pivotal role of DC, rather than macrophages, in the early immunoregulation of host response to murine cutaneous leishmaniasis.

6.1 Human studies

Research has begun to identify disease susceptibility and resistance factors in the human population (reviewed by Blackwell 1996). Not surprisingly, T cell and cytokine responses in humans are more complex and less polarised than they are in inbred strains of mice. Nevertheless, the murine model of leishmaniasis has led to

several important observations in humans. There is a clear requirement for IFN-γ to cure human disease, making IL-12 an attractive potential adjuvant for vaccination and therapy. Genetic susceptibility to different forms of leishmaniasis does appear to exist in humans and provides the basis for a relatively new area of intensive human research. .

6.2 Future Perspectives

The future in managing leishmaniasis is to develop vaccines using DC to treat and prevent the infection with the generation of long-lasting immunity. Protective effects have already been observed with some infections. DC pulsed with dead *Chlamydia trachomatis*, another intracellular pathogen, produced IL-12p40 and afforded protection to the female genital tract equivalent to that seen following live infection (Su, et al 1998). Similar protective effects have been observed after immunisation with microbe-pulsed DC in other models, including *Borrelia burgdorferi* (Mbow, et al 1997), murine lymphocytic choriomeningitis virus (Ludewig, et al 1998) and Toxoplasma (Bourguin, et al 1998). Heidrun Moll's group have also shown that susceptible mice could be protected against *L.major* infection following immunisation with *L.major* antigen pulsed LC.

DC have also been used in genetic vaccination strategies. DC isolated from the skin of gp63-DNA vaccinated mice were able to induce protection against *L.major* in naïve recipients (Walker, et al 1998). Even more novel and exciting is genetic manipulation of DC, by retroviral transduction, to impart constitutive IL-12

production which then augments DC capacity to stimulate a response to *Leishmania major* in vitro (Ahuja, et al 1998).

This study has also identified a potential role for DC in the search of non-peptide antigens shed by *L.major* which are presented via non-classical MHC pathways, as potential vaccine candidates. CD1 molecules have recently been shown to be involved in the presentation of lipoglycan, an antigen abundant on the surface of *Leishmania* promastigotes, to specific T cells (Porcelli and Modlin 1999).

The challenge now lies in refining our understanding of the DC response to *L.major* pathogens in order to identify optimal conditions for antigen presentation by DC and development of effective vaccines which are safe and produce long lasting immunity. Extensive research has shown that different DC subsets can lead to different functional outcomes. These subsets require further definition. How DC process antigen, particularly whether they are able to control parasite replication and if so, by what mechanisms, is still relatively unexplored. The feedback mechanisms from lymphocytes and other APC to activated DC which may modulate DC surface molecule expression and cytokine and chemokine production are as yet undefined. Such feedback could ultimately determine the nature of the adaptive immune response, particularly in determining whether tolerance or immunity develops. The role DC play in interacting and activating other APC in close proximity at the site of entry of the infectious parasite is also unexplored. The receptors and surface molecules involved in activation of the DC needs to be characterised as these may present molecular targets for the development of therapeutic interventions as well as antigens for DC vaccination research.

6.3 Concluding remarks

Due to the degree of human suffering caused by leishmaniasis, a great need exists to translate the advances in the understanding of the immune response and the pathogenesis of leishmaniasis into effective, affordable and practical methods for treatment and prevention of this disease. Knowledge of the immunoregulatory mechanisms the host uses for control of infection will eventually lead to better treatments. This was highlighted in this manuscript where the observation that NO is required for intramacrophage killing of parasites was translated into the search for NO-generating local therapies.

The conventional view that antigen processing and antigen presenting are relatively mutually exclusive functions of DC, depending on their degree of maturation, has been further challenged by this work. Delineation of DC subtypes with distinct characteristics and functions will provide a greater understanding of the role they play in the intricate intercellular interactions which determine disease outcomes. The studies performed in this thesis provide further evidence that DC play a critical and pivotal role in the pathogenesis of leishmaniasis. The development of the in vitro model used in this study has provided a valuable tool to further investigate antigen presentation in the immune response to leishmaniasis. Substitution of mast cells for DC in this model will allow study of the role of mast cells in leishmaniasis. Antigens derived from PM cultures can be assessed for their immunogenicity using this in vitro system, with a particular emphasis on non-peptide antigens and CD1 presentation. Results of this work and proposed studies using this model will add to

the groundswell of research to ultimately develop cytokine and DC-directed strategies for future treatment and prevention of leishmaniasis.

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APPENDIX

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